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ERYTHROCYTOSIS-PROMOTING ACTIVITY IN STAGNANT BLOOD AND IN BLOOD SUBJECTED TO LOW ATMOSPHERIC PRESSURE IN VITRO

by

EVA HIRSJÄRVI

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PREFACE

The subject of the present study crystallized during an earlier piece of work performed under the guidance of Professor Eeva Jalavisto, M.D. She has also supervised the present investigation, and has shown unfailing interest in it. I wish to express here my gratitude for her valuable advise and criticism, as well as for the encouragement I have received from her.

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Helsinki, 1. 3. 1953

Eva Hirsjärvi.

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INTRODUCTION

»La vie est le maintien d'un équilibre incessamment menacé.» (Bordet)

This definition of life includes the most characteristic properties of a living organism: continuous striving after a balance which is continuously threatened by numerous influences from its surroundings. The balance is, therefore, not of a static, but of a dynamic nature; incessant adaptation to new surroundings and new needs. The more adequate the adaptation mechanisms of an organism are, the better it is fitted for the battle of life.

The adaptive mechanisms of the highly developed mammalian organism are numerous and complicated. They allow the organism to meet successfully the demands of different conditions, and, to a certain degree, even to withstand the attacks of pathological processes.

The number of red blood corpuscles in organisms living in unchanging conditions is maintained at a fairly constant level. When, however, the need for oxygen carriers is increased, as, for instance, because of a deficient arterialisation of the blood in the lungs, of some disturbance in tissue respiration, or of an increase in the oxygen consumption, the erythropoietic organ is able to react with an increased activity and thus take part in the adaptation to the new conditions.

The subject of the present study is the mechanism responsible for the stimulation of the erythron in anoxic conditions.

Since the latter half of the last century it has been established that in men and test animals during a sojourn at high altitudes there is an increase in the oxygen capacity of the blood (P. Bert 1882) and in the number of red blood cells (Viault 1890). Mountain erythrocytosis has since been investigated by many authors, and

its nature has been a subject of much discussion. Though the rôle of some climatological factors, such as ultraviolet irradiation, has been emphasized as a part factor by Kestner (1921), Seyderhelm (1932) and Bockstahler (1936), the lowered oxygen tension in the inspired air is generally recognized as the chief cause of mountain erythrocytosis. This opinion is supported by the observation that an erythrocytosis similar to that due to high altitudes is also present in test subjects at sea level when inspiring either rarefied air (Regnard 1892, Schaumann and Rosenqvist 1898, Loewy and Heller 1933, and others), or gas mixtures of normal atmospheric pressure, but of a low oxygen partial pressure (Kuhn 1907, Brühl and Hanisch 1942).

Differing views have been taken regarding the nature of the high mountain erythrocytosis. A detailed account of the various theories on this subject is beyond the scope of the present study; only the main opinions forwarded will be mentioned.

Bunge (1895), Eimer (1929) and Asmussen and Nielsen (1945) are of the opinion that the rise in the red cell count is only apparent and is due to haemoconcentration. Schönholzer and Lüthi (1944) claim that the early erythrocytosis is due to haemoconcentration, but is later replaced by a real increase of the circulating red cells, caused by increased erythrocyte formation.

Emptying of the blood depots is suggested as the cause of high mountain erythrocytosis by Abderhalden and co-workers (1927), Backmund (1933) and Verzár and co-workers (1933), while Zunz and co-workers (1906) consider peripheral erythrocytosis to be due to redistribution of the red blood cells. Fick (1895) points out the possibility of the erythrocytosis being a result of increased longevity of the red cells. His opinion is supported by Heilmeyer, Rechnagel and Albus (1933) and Kaulberscz (1933), while according to Abderhalden and co-workers, Hurtado (1932) and Krupski and Almasy (1937) the increased age of erythrocytes is only a part factor causing the erythrocytosis.

Most authorities, however, consider high mountain erythrocytosis to be mainly due to increased production of red cells in the bone marrow. This opinion is based on examination of the peripheral blood as well as of the bone marrow of anoxic subjects. Several investigators (Egger 1893, Koeppe 1893, Schaumann and Rosenqvist, Hurtado etc.) observed a discrepancy between the

increase of the red cell count and the haemoglobin value at high altitudes, and interpret this observation as an indication that a new population of red cells has entered into the circulation. Increase of the red cell volume (Hurtado, Tallbott 1936, Schönholzer and Lüthi) and resistance (Hurtado, Wilbrandt and Herrmann 1944) is also well in accordance with this view. The increased percentage of reticulocytes and of nucleated red cells, in subjects living under low atmospheric pressures (Schauman and Rosenqvist, Barcroft and co-workers 1923, Drastich 1927, Goldbloom and Gottlieb 1930, Kaulberscz, Tallbott, Krupski and Almasy) is generally regarded as a sign of increased erythropoiesis. Studies of bone marrow most decisively prove the bone marrow origin of the anoxic erythrocytosis; Baló (1928), Kiyoshi (1928), Zalka (1931) and others report a marrow picture of increased activity in subjects living under a low oxygen partial pressure.

To summarize, the view that the high mountain erythrocytosis is due to increased activity of the erythropoietic organ seems well established.

The ability of bone marrow to release an increased amount of young red cells into the circulation quickly enough to account for early erythrocytosis which sets in immediately or a few days after the ascent has, however, been disputed. Several authorities, therefore, regard mountain erythrocytosis as a result of two mechanism: of an immediate emptying of blood depots, which is followed by the slower adaptation of bone marrow to a level of increased erythrocyte production. (Kuhn, Bürker 1911, Morawitz 1913, Krupski and Almasy, Vannotti and Markwalder 1939).

The natural consequence of low oxygen pressure in the inspired air, and thus the most probable and generally accepted cause of mountain erythrocytosis is the deficient arterialisation of blood in the lungs. This seems a valid enough explanation for erythrocytosis at high altitudes where the oxygen saturation of arterial blood is decreased considerably below the values at sea level. — Verzár and Vögtli (1945) report at 3,450 m an average arterial oxygen saturation of 84 %. The problem of erythrocytosis and arterial oxygen saturation at midaltitudes is more confusing. According to several authorities, sojourn at altitudes lower than 2,000 m where no deficiency of arterialisation of the blood, on the basis of theoretical calculus is known to exist, still promotes erythrocytosis

(Egger, Miescher 1893, Koeppe, Abderhalden, Terzioglu 1949). Several attempts to explain this discrepancy between the reaction of the erythropoietic organ and the lack of a recognized stimulus have been made. Miescher points out that the alveolar oxygen pressure needed to grant full arterialisation of the blood might be higher than expected on a theoretical basis, as the contact time of blood and alveolar air is limited. Further, the oxygen pressure in the poorer ventilated parts of lungs may be lower than in the better ventilated central parts, and these poorly ventilated parts might be responsible for the finer regulation of erythropoiesis. Schaumann and Rosenqvist suggest that the oxygen saturation of haemoglobin at a low oxygen pressure might involve some special energy requiring process. Morawitz points out that, even if the alveolar oxygen tension at high altitudes may be sufficient to maintain normal arterial oxygen saturation in resting subjects, exercise might yet cause a state of anoxia. Bürker and co-workers (1913) regard the want of oxygen in the tissues as the main stimulus for erythrocytosis; tissue anoxia, on the other hand, might be attributed partly to purely physical factors, such as congestion in the pulmonary circulation. Kroetz (1931) remarks that the arterial oxygen pressure, and thus the oxygen supply to the tissues is largely dependent on the conditions of gaseous exchange in the lungs, and that the alveolar oxygen tension is not a reliable measure for the oxygen saturation of blood. Van Liere (1942) emphasizes the rôle of the low carbon dioxide partial pressure in inspired air and in arterial blood, which causes a shift to the left in the oxygen dissociation curve of blood and thus prevents oxygen uptake by the tissues. He further points out that it is not the percentage of oxygen saturation, but particularly the oxygen pressure of the blood which is essential for the oxygen supply to the tissues.

The problem of arterial oxygen saturation and erythrocytosis in midaltitudes has recently been treated by Verzár and his coworkers in several papers. Verzár and Vögtli observed that the oxygen saturation of human subjects equals that stated at sea level (95 %) up to the altitude of 1,800 m, but decreases to 84 % at 3,450 m — a result which is well in accordance with the dissociation curve of blood by Barcroft (1914) as well as with later dissociation curves obtained by more modern methods (Drabkin 1949). In a later paper, Wang, Wirz and Verzár (1951) confirm the result that at 1,800 m

there is no significant decrease in the oxygen saturation of human subjects whereas rabbits at this altitude show an oxygen saturation of only 84%. Red cell and haemoglobin determinations parallel these results. Doetsch, Verzár and Vögtli (1945) observed no significant increase in human subjects at 1,800 m. On the other hand, rabbits taken to this altitude showed signs of increased erythropoesis (Wang and Verzár 1949). Also in children at 1,800 m a slight but still significant increase in the red cell count, as compared to the values at sea level, was observed. These results, on the one hand, confirm the rôle of lowered oxygen saturation of arterial blood as the cause of mountain erythrocytosis. On the other hand, erythrocytosis in human adults at mid-altitudes is dubious, and, even if present in some cases, cannot reasonably be attributed to lowered arterial oxygen saturation, but rather to a general stimulating effect of some other factors (e.g. ultraviolet irradiation, light, cold).

As regards the *mechanism* of the anoxic erythrocytosis, the theory that the local want of oxygen in bone marrow directly stimulates the erythropoiesis has been widely accepted. This conception is mainly based on the observation that polycythaemia is a constant symptom in diseases with impaired bone marrow circulation (see Davis 1941). F. Müller (1901 and 1910) attempted to prove the direct stimulation by clamping the nutriant tibial artery and thus causing anoxia in the bone marrow. After an obstruction of 20 min.—$1^1/_2$ hours he was able to detect nucleated red cells in the blood of the nutriant tibial vein. Nucleated red cells also appeared after removal of blood or when breathing air with a low oxygen partial pressure. Reusch (1911) repeated Müller's experiments in a modified way by obstructing the circulation in one hind limb of a rabbit for $1^1/_2$ hours on several successive days. He could not observe any erythrocytosis after the treatment, nor was there any difference in the bone marrow of the treated and untreated leg when the animal was killed. Reusch concludes that bone marrow anoxia does not necessarily cause erythropoietic stimulation. In 1950 Benkö, Petri, Eisner, Kardos, Szabo, Bentzik and Hetenyi observed a general increase in the erythrocyte count of peripheral blood following the ligation of the femoral artery of dog, and the same investigators in another paper (see Petri and co-workers) showed by a crossed circulation experiment that the erythrocytosis

is transmitted by a humoral, and not by a nervous route. According to these authors bone marrow anoxia gives rise to the formation of some humoral bone marrow stimulating factor.

Direct study of the bone marrow in vivo and in vitro indicates, however, that a low oxygen pressure in the bone marrow must be disregarded as an erythropoietic stimulus.

Warren (1941) studied, in two separate papers, the respiratory metabolism of bone marrow kept in vitro under low oxygen pressures, but could never observe any increase in the rate of respiration, whereas in bone marrow from rabbits exposed previously to low atmospheric pressure, the rate of respiration was constantly increased. On the basis of these results, Warren writes: »I cannot see how low oxygen pressure could act directly as a stimulus to bone marrow metabolism.» Warren further states that the increase in the bone marrow respiration of anoxic animals is effected neither by the nervous system, nor by any serum factor, as the bone marrow respiration is similarly increased in a denervated and in a control limb of anoxic animals, and as, on the other hand, suspending bone marrow slices in serum of anoxic animals does not influence the respiration. The results of Rosin and Rachmilewitz (1948) and of Magnussen (1949) are also in contrast to the theory that local bone marrow anoxia acts as an erythropoietic stimulus; Rosin and Rachmilewitz could not discover any signs of increased activity in bone marrow cultures kept under low oxygen pressures, while cultures in less than 5% of oxygen showed markedly diminished activity. According to Magnussen maximal erythrocyte production in the bone marrow takes place in an oxygen partial pressure of 10—30%, while complete arrest of erythropoiesis is observed at 2.5% of oxygen. Grant (1947 and 1948) when measuring the oxygen saturation of the bone marrow blood in dogs, made anaemic by bleeding, found it to be of the same percentage as in normal dogs, and accordingly concludes that low local oxygen pressure is not the only stimulus for bone marrow erythropoiesis. Berk and co-workers (1948) measured the oxygen saturation of bone marrow blood of normal human subjects, and of patients with polycythaemia vera, with secondary polycythaemia, and with chronic anaemia. They found the oxygen saturation in polycythaemia vera as well as in anaemia to be normal, whereas in patients with secondary polycythaemia (cardiac or pulmonary disease)

the marrow oxygen saturation was below the normal limit. These investigators, however, consider the result as due rather to technical errors — most probably in collecting the samples of marrow blood — in the groups comprising patients with polycythaemia vera and anaemia, than as a proof against the local bone marrow anoxia as an erythropoietic stimulus. A recent study by Aslaldi, Bernadelli and Rebando (1952) on the effect of reduced atmospheric pressure on bone marrow implantates also shows that hypoxia has a depressing effect on bone marrow erythropoiesis in vitro.

On the basis of the evidence given above, the rôle of low oxygen pressure in the bone marrow as the stimulus for erythropoiesis seems unlikely and therefore humoral, nervous or nervous-humoral transmission of the anoxic erythrocytosis must be assumed.

Apart from the crossed circulation experiments of Petri and co-workers, the existence of a humoral mechanism in anoxic erythrocytosis has also been demonstrated by injection experiments. Already in the beginning of this century (1906) Carnot and Deflandre claimed the presence of some erythropoietically active substance in the serum of anaemic organisms. They rendered rabbits anaemic by bleeding and on the next day, when the regeneration of the blood was well started, injected samples of 10—15 cc of the anaemic serum into untreated test rabbits. The latters constantly responded to the injection with a rise in the number of red blood cells, well over 1 Mill./cu.mm. The erythropoietic activity of the serum, according to Carnot and Deflandre, is directly proportional to the signs of regeneration of the donor animal. However, if the animal is bled several times at short intervals, the regeneration of its blood is suppressed and its serum simultaneously loses its erythropoietic activity. Gerhardt (1910) made an attempt to reproduce the experiments of Carnot and Deflandre, but failed to observe any reactions. On the other hand, Gerhardt reports an injection of 5 cc of defibrinated blood to have improved the blood values of anaemic patients. In Gibelli's (1911) experiments anaemic as well as normal rabbits were used as recipients. According to his results, injections of anaemic sera into normal rabbits were always followed with a marked erythrocytosis, while anaemic recipients failed to react unless there had been an interval of 8—10 days between the bleeding and the injection. Serum from animals with infections had no effect on the recipients red cell count, neither was there any effect when active

serum was injected into infected or starved animals. P. Th. Müller (1912) when using heterologous serum (donor: guinea pig, recipient: mouse) observed a decrease in the red cell count of the recipient after an injection of serum from a normal animal. If, however, the donor was made anaemic by bleeding, the serum injections caused only a slight decrease or, in some recipients, no change in the red cell level. Only a slight decrease in the recipients red cells was observed when the serum injected was drawn from animals rendered anoxic by exposure to low atmospheric pressure. Müller concludes that the effect of Carnot's serum is not due to bleeding as such, but to the corollary anoxia. Morawitz (1913) doubts the rôle of anoxia as such in the stimulation of erythropoiesis, and considers the existence of some chemical agent of the bone marrow stimulating activity as highly probable. When repeating Carnot's and Deflandre's experiments, however, he only observed elevation of some 100,000 Er/cu.mm in the recipient. Using slightly anaemic recipients the result was still the same. The very large increase in the recipients red cell count after injection of only 10—15 cc of serum claimed by Carnot and Deflandre strikes Morawitz as rather unexpected, considering the relatively more moderate reaction of the donor animal.

The investigations of Förster (1924) have been carried out with more test animals, and the results are recorded in more detail than those of earlier investigators, and consequently they are considered to be more convincing. His results on the effect of anoxia and of anoxic serum on erythropoiesis, summarized, are as follows: When an anaemic animal is exposed to a low atmospheric pressure, or receives injections of serum from an animal rendered anoxic by exposure to low atmospheric pressure, a marked stimulation of the regeneration is observed. When the animal is subjected to low pressure, this acts as a stronger erythropoietic stimulus than an injection of anoxic serum. Insufflation of oxygen through the anoxic serum increases the erythropoietic activity. Serum from normal animals or foreign protein (milk) is ineffective as a stimulus of regeneration. If, on the other hand, normal recipients are used, no erythrocytosis is observed after injection of either normal or anoxic serum. In view of the negative results after injection of normal serum or milk, Förster considers erythrocytosis which follows the administration of anoxic serum as due to some specific substance formed in

the bone marrow during exposure to low atmospheric pressure and delivered into the circulating blood.

In the following decade only little interest was given to the haemopoietin investigation. This is probably due to the fact that several investigators have obtained negative results when attempting to prove the existence of Carnot's haemopoetin and thus the humoral transmission of the anoxic erythrocytosis has generally been considered as a speculative theory without sufficient experimental support. Leffkowitz and Leffkowitz (1926) report that injections of normal as well as anaemic serum shorten the regeneration time of bled animals, while normal test animals may react to a normal as well as to an anaemic serum either with an elevation or a fall in the red cell count. Gordon and Dubin (1934) failed to demonstrate any erythropoietic activity either in an anaemic or in an anoxic (»low pressure») serum. Feenders (1936), after injecting anaemic serum, could discover only moderate increases in the recipients' red cell level which never exceeded the range of normal daily variations. These investigations have, however, been carried out with few test animals, and their results cannot be regarded as decisive proof against the existence of an erythrocytosis promoting factor in anoxic blood, but might be, at least partly, attributed to the influence of environmental changes on the reactivity of donor as well as recipient animals. It should also be noted that Gordon and Dubin did not inject the serum samples into the recipients immediately but usually on the second day when the activity of the serum might already have vanished (see Klingelhöffer 1950).

In recent times, again, new interest in the haemopoietin problem has been aroused in different laboratories almost simultaneously. Experiments carried out on large series of test subjects have been published and even some information concerning the properties of the active substances has been added to our knowledge. In 1939 Loeschke and Schwartzer studied the erythropoietic activity of blood from new born babies. Their work was based on the hypothesis that, as the conditions during intrauterine life closely correspond to those during a sojourn at high altitudes (low arterial oxygen saturation and simultaneously increased oxygen capacity of the blood) some erythropoiesis promoting substance should be present in foetal as well as in »high mountain» blood. In accordance with this hypothesis they succeeded in showing that normal test

rabbits reacted to the injection of serum drawn from the umbilical cord of new born infants or from young babies during the first weeks of life with an erythrocytosis similar to that caused by injection of »high mountain» serum, while serum from older, icteric babies was erythropoietically ineffective. The haemopoietic effect of plasma from acutely and chronically anoxic organisms on normal test animals was compared by Bonsdorff and Jalavisto (1949). As acutely anoxic donors, rabbits exposed to lowered atmospheric pressure were used, while the chronically anoxic group comprised patients with cardiac failures. Plasma from both groups caused a moderate, but yet statistically significant increase in the recipients' red cell count, in the percentage of reticulocytes and in haemoglobin. A polycythaemic reaction of the same magnitude was observed by Bonsdorff (1949) in rabbits given injections of plasma drawn from sheep foetuses during the latter part of gestation.

The erythrocytosis promoting effect of chronically anoxic (anaemic) serum has also been confirmed by bone marrow biopsy. Krumdieck (1943) reports an increase in the erythrogenic/myelogenic cell ratio (E/M ratio) in rabbits following injection of serum from chronically anaemic animals, whereas there was no difference in the E/M ratio in recipients injected with serum from acutely anaemic animals as compared to those receiving injections of normal serum.

Regarding the nature and properties of the erythropoietic substance, little is known as yet. Some approaches to the problem from various sides have been made, but the results obtained hitherto are not very convincing. Carnot and Deflandre already tested the thermostability of their »anaemic haemopoietin», and claim a complete loss of haemopoietic activity when the serum is heated to 55° C. P. Th. Müller, on the other hand, reports that anaemic serum retains full activity on heating to 56° C. Döring and Loeschke (1949), on the basis of results confirmed by statistical calculus, state that the serum from the umbilical cord preserves its activity when heated to 67° C.

The effect of atmospheric oxygen on »haemopoietin» was investigated by Klingelhöffer (1950). His experiments indicate that insufflation for 15 minutes with a mixture of nitrogen and oxygen, containing more than 8 % of oxygen is sufficient to abolish the haemopoietic activity of a formerly active plasma sample; gas

mixtures with 5% of oxygen diminished the activity, while in samples insufflated with gas mixtures of less than 3.8% of oxygen full activity was preserved. This result leads the author to question, whether the failure to demonstrate the haemopoietic activity of anoxic serum might depend on aerobic manipulation of the samples. Also Loeschke (1950) emphasizes the importance of anaerobic manipulation. He further suggests that not the formation of haemopoietin, but the failure to inactivate it, is essential for anoxic erythrocytosis.

The correlation of anoxic haemopoietins to the spleen and the reticuloendothelial system, as well as their physical properties, was treated by Yu-Tin Tei (1938). He used as anoxic donors rabbits rendered anaemic by bleeding, by phenylhydrazine poisoning, by garlic feeding, by blockading the reticuloendothelial system (RES) with Indian ink or by irradiation with ultraviolet rays, or polycythaemic rabbits which had been subjected to low atmospheric pressure. In each of these conditions, simultaneously with the appearence of signs of increased erythropoiesis in the donor animal, the serum gains haemopoietic activity, which can be demonstrated by injecting samples of serum either into normal or into bled animals. Splenectomized animals, when used as recipients, react to the injection of active sera with a higher degree of erythrocytosis than untreated recipients. The formation of the active substance is not affected by the removal of the spleen in animals made anaemic by excessive ultraviolet irradiation, garlic feeding or phenylhydrazine poisoning, while in animals rendered either anaemic by blockading the reticulo-endothelial system or polycythaemic by exposure to low pressure, splenectomy prevents the formation of the active substance. A normally functioning RES is essential for a polycythaemic response of the recipient since no erythrocytosis occurred in animals with a previously impaired RES after injection of the active substance. Donors with an impaired RES failed to produce the haemopoietically active agent when subjected to low pressure, while, on the other hand, in anaemic animals with a blockaded RES the haemopoietin formation was normal. Tei further claims that the active substances present in the serum in different kinds of anaemia and polycythaemia differ from each other in some chemical and physico-chemical properties: the active substance in sera from animals either exposed to low atmospheric

pressure or fed with garlic are globulin-like in nature and are destroyed at 54° C, while the active agents in sera from animals rendered anaemic by ultraviolet irradiation, venesection, phenylhydrazine injections or blockading of the RES, are thermostable and of lipid nature.

The close correlation between the production and destruction of red blood cells manifested in the organisms ability to maintain a constant red cell level even when one of the processes is stimulated or depressed, by a compensatory depression or stimulation of the other process, has led to the question whether increased red cell destruction might be the natural stimulus of erythropoiesis. This theory has been forwarded and defended by Verzár and his school. The investigations of Verzár have followed two main lines; studies of the serum bilirubin in subjects with increased erythrocyte formation, and studies of the erythropoietic activity of the destruction products of haemoglobin.

Though during high mountain expeditions increased serum bilirubin in human subjects and in some species of animals has been observed, it still remains unsolved whether bilirubinaemia is the cause of the erythrocytosis, or merely a consequence of a new, higher level of both erythrocyte production and destruction. Giannini (1929) claims that in some species the serum bilirubin is increased during the sojourn at high altitudes, while in others an elevated bilirubin level is observed only after descent — a sign of increased red cell destruction. Goldbloom and Gottlieb (1930) could not discover any increase in the serum bilirubin in guinea pigs rendered polycythaemic by exposure to low atmospheric pressure. Hurtado (1932) observed an increase of plasma bilirubin of polycythaemic human subjects at high altitudes, but does not consider the bilirubinaemia to be the cause of polycythaemia, but rather to indicate a higher rate of red cell destruction, which is caused by a primary increase in red cell production. According to Krupski and Almasy (1937), no increase in the bilirubin content of serum takes place either during or after a sojourn at high altitudes, and Vannotti and Markwalder (1939) report bilirubinaemia only in unacclimatized subjects when working at high altitudes. Even the results of Verzár and co-workers (1933, 1945) are not quite consistent as regards high mountain bilirubinaemia: while some subjects show an increase in the serum bilirubin immediately after ascent,

others develop bilirubinaemia only later, after the increase of erythrocytes has already taken place. Verzár admits that his experiments do not allow the conclusion that increased serum bilirubin is responsible for mountain erythrocytosis.

To obtain more precise knowledge of the relation of red cell production and destruction during anoxia, the excretion of the destruction products should also be considered.

Rich (1930) remarks that increased red cell destruction can never alone account for haemolytic icterus; impairment of liver cell function must be involved. Heilmayer (1931) also points out that increases in the serum bilirubin as well as in the urobilinogen excretion are dependent on two different factors, namely the rate of red cell destruction and the function of the liver. When studying red cell destruction at high altitudes, Heilmayer, Recknagel and Albus (1933) found a decrease in the urobilin excretion, which is interpreted as indicating a decreased erythrocyte destruction, and, consequently, an increase in the age of the erythrocytes. Merino (1950), working on the same subject, remarks that if the increased level of serum bilirubin were solely due to an increased rate of red cell destruction, the excretion of urobilinogen should also be proportionally increased. This is not the case with his test subjects, for the values for urobilinogen excretion never exceed those expected on the basis of the total amount of the circulating haemoglobin. The slight bilirubinaemia, encountered in some cases, is, according to Merino, due to diminished excretion of bilirubin by the liver, probably because of the anoxic state.

Reissmann, Burkhardt and Hoelscher (1952) studied the bile pigment excretion by an internal bile fistula in anoxic polycythaemia. They observed an increase in bile excretion during the first weeks of anoxia, which, however, showed no significant correlation to the erythrocyte response.

The investigations of the Verzár school on the effect of erythrocyte destruction products on erythropoiesis have been published in a series of papers, in which bilirubin, biliverdin, haematin and haemoglobin are claimed to stimulate the red cell formation. (Verzár and Zih 1929, Bencsik, Gaspár, Verzár and Zih 1930, Zih 1930 and 1939). The erythropoietic effect is observed when administering bilirubin orally as well as intravenously, intramuscularly or subcutaneously. Peroral bilirubin doses as small as 0.5 mg

for rabbits, resp. 0.25 mg for rats and 0.01 mg for mice, proved already effective, while larger amounts often caused erythropenia. Zih (1930) claims further that serum from anaemic animals promotes erythrocytosis only in cases in which the sample contains haemoglobin, while haemoglobin free samples have no effect. Also haemolysed samples of normal sera have erythropoietic activity. — The results of Verzár and his co-workers have, however, been severely criticized by later investigators. It has been pointed out that their results are very uneven, and, if treated statistically, would not allow any serious conclusions.

Results indicating an erythropoietic effect of the products of erythrocyte destruction have, however, also been obtained by investigators outside the Verzár school, though there is much disagreement between the results of different investigators. In 1911 Kcpinow reported the erythrocyte lipids to have a favourable effect on the regeneration of anaemic animals. Kerti and Stengel (1929 and 1930) claim a decrease in the red cell count and haemoglobin of normal test animals after administration of ultraviolet irradiated as well as untreated bilirubin. The red cell level was, however, soon normalized after administration of irradiated bilirubin, while after untreated bilirubin the erythrocyte count remained at a low level for a longer period. By prolonged administration of untreated or irradiated bilirubin the initial decrease was followed by an increase up to normal values, and then again, by a drop. The same undulating form of the erythrocyte curve is also obtained when using anaemic recipients, but instead of the initial drop there is an initial rise in the red cell and haemoglobin level. Bile acids were stated to have a similar effect on the red cells and haemoglobin. According to the investigators, the initial decrease might be explained by the strong haemolysing property of bilirubin, while no explanation is given for the initial rise in erythrocytes in anaemic animals, nor for the shape of the erythrocyte curve. Popper (1930), after intravenous bilirubin injections into human subjects, never stated any increase in the red cell count, though the haemoglobin level was always elevated. In Fellingers (1932) experiments bilirubin was administered intracardially to normal and anaemic rats. Approximately one half of his subjects responded to the injection with a rise in the red cells of short duration, while by prolonged administration the erythrocyte values remained high

as long as the treatment was continued. The regeneration of anaemic animals was also favourably influenced by bilirubin treatment. Miller and Rhodas (1934) studied the peripheral blood and the bone marrow following injections of haemoglobin into anaemic rabbits. They observed an »enormously increased erythropoiesis», manifested as well in a rapid elevation of the haemoglobin level as in a picture of increased erythropoiesis in the bone marrow. Patek and Minot (1934) observed, in anaemic patients, signs of speeded regeneration during combined bile pigment and iron treatment, probably due, according to the investigators, to the furnishing of building material in the formation of haemoglobin. Schernhardt (1939) claims that small amounts of haemolysed blood cause an erythrocytosis, large amounts an erythropenia in the recipient. Bomford (1940) made an attempt to decide whether the erythrocytosis promoting effect of bilirubin was due to specific stimulation of erythropoiesis or to reutilisation of the breakdown products in the haemoglobin synthesis. For this purpose he compared the regeneration in anaemic dogs treated with bilirubin resp. bilirubin and iron combined. In both cases increased regeneration was noted, but the effect was stronger when iron was given too. The author interprets this result as to indicate that bilirubin was utilized in the haemoglobin synthesis, but need not act as a specific stimulant for erythropoiesis.

Jeney (1933), when incubating bone marrow with various substances, found that haematin and some related compounds (haematoporphyrin, haematinic acid, pyrrhole), when combined with iron, stimulated the formation of normoblasts, while by incubation with globin primitive red cells were formed.

In the work of Lambrechts and Nizet (1947) the correlation of the destruction products of haemoglobin to haemopoiesis was studied by comparing the appearance of the signs of increased haemolysis and of increased haemopoietic activity. By means of phenylhydrazine injections haemolysis was induced in the test animals, which could be easily followed, as the phenylhydrazine poisoned red cells are distinguished by the granulae of Heinz, and thus the disappearance of the granulated cells gives a measure for haemolysis. It was shown that the rapid decrease in the number of the granulated cells, occurring on the 3rd or 4th day after the injection, was followed within one day by a reticulocyte peak indicating

increased erythropoiesis. In order to decide whether the stimulation of erythropoiesis was due to increased destruction of erythrocytes or to anoxia consecutive with the disappearance of the poisoned cells, normal dogs were given transfusions of phenylhydrazine poisoned blood. Even in these normal dogs, the disappearance of the granulated cells preceeded the reticulocyte peak. On the basis of this result the investigators claim the erythropoiesis stimulating effect of red cell destruction products.

As further indirect evidence of the erythropoiesis stimulating effect of the destruction products of the red blood cells it has been pointed out that anaemias due to internal bleeding regenerate quicker than those due to removal of blood from the body (Itami 1909, Gerhardt 1910, Morawitz 1913). The observation of Boycott and Oakley (1933) that, after transfusions, a fall in haemoglobin is followed by a rise in the percentage of reticulocytes, might also be interpreted in favour of the importance of the destruction products of haemoglobin for blood regeneration.

The correlation of the anoxic haemopoietins to the desturction products of haemoglobin has been studied by the Göttingen school. Döring (1948) compared the erythropoietic effect of plasma and washed cells of anoxic blood: while injection of plasma resulted in a statistically confirmed increase in the recipients' red cell count, injections of red cells suspended in physiological saline had no effect.

Summarizing the results cited above, the erythropoietic effect of destruction products of erythrocytes cannot be wholly denied. On the other hand, the question can hardly be regarded as satisfactorily settled, as the results of different authors are disagreeing. The reliability of those experiments of the Verzár school, in which bilirubin and its related compounds administered orally were stated to promote erythrocytosis, seems rather doubtful, as, according to the common conception, bilirubin is not resorbed from the intestine (Whooper and Whipple 1917, Bollman, Sheard and Mann 1926, Scholderer 1933, see also Lemberg and Legge 1950), and, on the other hand, the normal diet already contains bilirubin or substances which are converted into bilirubin, in considerable amounts. It might also be remarked that erythropoietic reactions might hardly be expected after administration of bilirubin in amounts which do not exceed the amount of bilirubin contained normally in the

serum. On the other hand, the fact that even small amounts of bilirubin cause erythrocytosis, would indicate that bilirubin acts as a stimulant for the bone marrow, for, in case it would only be used as material in the haemoglobin synthesis, larger amounts would be required to cause a marked elevation in the circulating haemoglobin.

The rôle of the spleen in the destruction of the red blood cells has long been acknowledged. Besides this activity, quite the opposite function has also been attributed to the spleen, i.e. the production of a haemopoietic hormone.

The effect of splenectomy and blockading of the reticuloendothelial system on the erythropoiesis has been studied by several investigators, and many differing opinions on the subject have been forwarded. Drastich (1927) and Beyne, Binet and Strumza (1934) report that splenectomized animals, resp. animals with a clamped lienal vein, do not respond to anoxia with erythrocytosis. According to Zalka (1931), the blockade of the reticuloendothelial system prevents the peripheral erythrocytosis in anoxic animals, while bone marrow biopsy, however, shows a picture of proliferation. Ruhenstroh-Bauer and Maier (1952) observed that the restoration of the erythrocyte number towards the normal after bleeding occurs similarly in normal and splenectomized animals, whereas the reticulocyte count remains lower in splenectomized subjects. Contradictory to these results is the statement of Gabathuler (1929) that the anoxic erythrocytosis is more pronounced in splenectomized than normal animals, and the observation of Gordon and Kleinberg (1939) that splenectomy does not change the animals reactivity to anoxia.

The results on the correlation of the spleen or spleen extracts to anoxic haemopoietins are also rather inconsistent. The pioneers of haemopoietin investigation, Carnot and Dflandre, never investigated the effect of the spleen on the activity of the anaemic serum, but they report that spleen extracts from anaemic as well as normal animals have no effect on blood formation. Ascher and Nakao (1925), studying the correlation of haemopoietins to some endocrine glands and spleen, observed the reactivity of the test animals to haemopoietins, which is lost after removal of thyroid and thymus, to be restored if the spleen is removed as well. According to Gabathuler, the concentration of anoxic haemopoietins reaches a higher

level in the blood of splenectomized test animals as compared with untreated animals, while, on the other hand, Krähenbühl (1933) reports blockading of the reticuloendothelial system to prevent the formation of the haemopoietins.

Downs and Eddy (1920 and 1922) have forwarded the hypothesis that the destruction products of erythrocytes are utilized by the spleen for the formation of some bone marrow stimulating agent. Leake and Bacon (1924) observed erythrocytosis in test animals following combined injections of spleen and bone marrow extracts. According to their theory, an inactive precursor of the haemopoietin is formed in the spleen and afterwards converted into the active form in the bone marrow. Leffkowitz and Leffkowitz (1926) observed an erythrocytosis after administration of spleen extracts and claim the existence of a special haemopoietic hormon in the spleen. According to Kokas (1926) extracts of spleen and bone marrow contain haemolytic as well as haemopoietic substances, and Zih (1928) reports that the effect of splenic extracts is dependent on the doses, small doses result in erythrocytosis, large doses in erythropenia. In the experiments of Bock and Frenzel (1938) the venous blood flow from the spleen was prevented from getting into the liver, by conducting it directly into the vena cava. In these experimental conditions erythropoiesis was found to be depressed. The investigators consider this effect due to an erythropoiesis depressing substance formed in the spleen being brought into the bone marrow in concentrations stronger than in normal conditions when a part of the substance is inactivated in the liver. Jombres (1939) completed Bocks and Frenzels investigations by studying the bone marrow in the same experimental conditions. He states that neither the formation nor the ripening of the red blood cells is disturbed, but the cells are not delivered into the circulation normally. Ruhenstroh-Bauer (1950, see also later) suggests the spleen as the place of formation of the bone marrow stimulating agent in anoxia. In a later paper, however, Ruhenstroh-Bauer and Maier conclude that though the rôle of the spleen in the regulation of normal erythropoiesis is probable, it still is not proved.

Some erythropoietic activity has been attributed also to the liver. Villa and Sala (1937) succeeded in inducing a reticulocytosis in test animals by injections of plasma from the hepatic vein, while plasma from the hepatic portal vein as well as from the vena cava

was ineffective. Beer (1942, see also later) claims an erythropoietic hormone to be formed in the liver after stimulation of the splanchnic nerve.

In addition to the works referred to above in other connections, numerous papers have been published on the effect of endocrine glands on anoxic erythrocytosis and heamopoietins. Though the present writer will not attempt to discuss these results in detail, some theories concerning the correlation of hormones to anoxic erythropoietins will be mentioned. (For a detailed literature review see Grant and Root 1952).

The erythropoiesis stimulating effect of thyroid extracts on the one side, and the depression of erythropoiesis after thyroidectomia on the other side, has been established by several authorities (Thåddea 1932, Heilmayer 1933, Beyne, Binet and Strumza 1934, Thaddea and Valy 1934, Nizet 1948). Mansfeld advanced his theory on the influence of thyroid on erythropoiesis in 1913. According to him, an intact and normally functioning thyroid gland is needed in order that the test animal would react with an erythrocytosis to anoxia or to anoxic haemopoietins. Thyroid extracts, on the other hand do not promote erythrocytosis comparable to that caused by haemopoietins. Gutstein (1921) forwarded the opinion that anoxia would primarily stimulate the secretory function of the thyroid gland, and that the increased thyroid secretion was responsible for the anoxic polycythaemia. This interpretation is based on the similarity of the bone marrow picture in morb. Basedow and in different kinds of anoxia.

In contrast to the conception of these authorities that the thyroid gland is indispensable for the development of the anoxic polycythaemia, Meyer, Thewlish and Rusch (1940) have found that normal and thyroidectomized animals respond to anoxia with an erythrocytosis of the same magnitude.

Other endocrine glands have also been claimed to have an influence on the anoxic erythrocytosis. Stewart and co-workers (1935) report that hypophysectomized rats subjected to an atmospheric pressure of 422 mm Hg show neither reticulocytosis nor bone marrow hyperplasia while in normal animals reticulocytosis and marrow hyperplasia occur. Feigin and Gordon (1950) claim that, while hypophysectomized rats at 422 mm Hg show no signs of increased erythropoiesis, further reduction of

the pressure to 321 mm Hg causes an increase in the erythropoietic activity. This result is attributed to the decreased metabolic rate of the hypophysectomized animals; a fall of the pressure to 422 mm Hg is not sufficient to produce an oxygen lack in hypophysectomized animals as it does in normal ones, and thus there is no stimulus for increased erythropoietic activity.

The influence of the nervous system on erythropoiesis is generally recognised. For literature review the reader is referred to Hoff 1938 and Grant and Root. Only some investigations dealing with the effect of the nervous system on anoxic erythrocytosis, and with the nervous-humoral regulation of erythropoiesis, will be mentioned here. — Warren states that increased bone marrow respiration and erythroid hyperplasia of anoxic animals is not dependent on integrity of the nervous system, and Petri and co-workers showed by crossed circulation technique that anoxic erythrocytosis is not transmitted by nervous route.

Beer investigated the humoral transmission of nervous stimulation of erythropoiesis by using two parabiosis animals. He showed that nervous stimulation of one parabiote caused an erythrocytosis of the same magnitude in both animals, and accordingly presumes the stimulation to be transmitted by some humoral agent, which, in his opinion, is formed in the liver. Ruhenstroth-Bauer used the same experimental arrangement to test the existence of the anoxic haemopoietin. Both parabiosis animals reacted with a reticulocytosis of the same magnitude on the removal of blood from one animal. The author's interpretation of this result is that anoxia acts primarily on the brain cells, whence the effect is transmitted by some humoral way to the spleen which is the place of formation of bone marrow stimulating agent (central way). Or, if the nervous elements should be damaged, the effect of anoxia might be transmitted by some unknown peripheral humoral way to the spleen, whence, again, the stimulation is transferred to the bone marrow.

THE PRESENT PROBLEM

As evident from the abovesaid, the question of the anoxic haemopoietins is, as yet, unsolved for a considerable part. The possibilities to approach the problem are many, and the choice must be largely based on practical consideration of material and methods available.

The purpose of the present study is to obtain some more detailed information on the conditions required for the formation of anoxic haemopoietins.

Until now the formation of haemopoietins has been studied in a whole organism. There is, however, always the difficulty of controlling the numerous other factors which are claimed to influence the red cell level, e.g. the nervous system, the reculo-endothelial system, spleen, endocrine glands. If, however, the formation of haemopoietins could be demonstrated in conditions, where the influence of these factors were minimized or excluded, further study of the problem would be greatly simplified. In the two series of experiments to be presented the formation of haemopoietins in such simplified conditions, in local venous anoxaemia and in blood in vitro, has been studied.

PART I

EFFECT OF LOCAL ANOXAEMIA ON THE PERIPHERAL BLOOD PICTURE

Literature on the effect of local anoxaemia on blood formation is scarce. According to the writers knowledge, the only report on the question dates back to Reusch (1911). His experiments were actually planned for the purpose of studying erythropoiesis in anoxic bone marrow, but, as the anoxia was induced by obstructing the circulation in a whole limb, venous anoxaemia in the limb must be counted upon as well. Reusch failed to observe any signs of increased erythropoiesis in the peripheral blood or in the bone marrow following the treatment. This result, however, can hardly be regarded as decisive, as the experiments were only performed on two rabbits, and no information is given as to the pressure used to induce obstruction.

Therefore, a more detailed study, using more test subjects, of the effect of local venous obstruction, induced by a known pressure, on the peripheral blood count was considered necessary.

TEST SUBJECTS AND METHODS

The subjects used were 25 healthy adults of both sexes, and 30 children warded at the Children's Clinic and diagnosed as having various complaints. The children were chosen so as to exclude troubles of blood or blood-forming organs, circulatory and respiratory systems, and infectious diseases. The age, diagnosis and blood counts of the children are given in table I_1.

The erythrocyte count of the test subjects varied from 3.3 to 5.8 Mill./cu.mm. Haemoglobin values ranged from 46 to 96 Sahli grades, and the percentage of reticulocytes from 0.4 to 2.0. As these values indicate, most of the children were suffering from a slight anaemia which was probably due to lowered general condition.

The samples of capillary blood for enumeration of red and white corpuscles and reticulocytes, and for determination of haemoglobin, were taken from the finger tip of the test subjects, from the left and right hand on alternate days. Venous blood for determination of the blood oxygen and serum bilirubin and for measurement of the packed cell volume and the red cell diameter was drawn from the cubital vein with the aid of as little stasis as possible. The blood samples for gas analyses were collected into paraffinized syringes containing a few crystals of sodium oxalate. Heparin was used as an anticoagulant for the samples for packed cell volume and mean erythrocyte diameter, while the samples for estimation of the serum bilirubin were collected into empty dry test tubes.

The enumeration of the red blood cells was performed in a Bürker counting chamber; for each enumeration 16 small squares were counted. Double determinations were made occasionally. The white cells were also counted in the Bürker chamber, 16 large squares each time. The haemoglobin values were determined by the acid haematin method of Sahli. For estimation of the percentage of reticulocytes, in the former part of the work, a fresh drop of blood was placed on a dry slide, dyed with brilliant cresyl blue, and a cover glass was placed on the drop. The count was performed after 15—20 minutes. For each determination 1,000 red cells were counted. In the latter part of the experiments, comprising the majority of the adult test subjects, a supravital staining method was used: a drop of 1% brilliant cresyl blue was mixed on a slide with a drop of blood and a smear was made of the mixture. After fixing with methyl alcohol, the slide was superstained with Giemsa solution for 20 minutes. This procedure allowed the slides to be preserved for a longer period and permitted re-counting afterwards, when needed.

The oxygen content, oxygen capacity and the percentage of oxygen saturation of the venous blood were determined by the ferricyanide method of Haldane and Priestley modified by Courtice and Douglas (1947). Double determinations were performed, the average difference of the double determinations being, for the percentage of oxygen saturation, 1.93 ± 0.35 % and for the oxygen capacity 0.75 ± 0.14 Vol. %. The method was chosen in view of it giving not only the oxygen content, but also the oxygen capacity and the percentage of oxygen saturation of the blood without any further manipulation. The precision of the method was considered to be of minor consequence, as the changes to be expected in the oxygen saturation were considerably greater than the average error of the method.

In order to obtain serum for the bilirubin determinations, the blood sample was allowed to clot for 1 hour and then centrifugalized. The bilirubin content of serum was estimated by the method of Jendrassik and Cleghorn (1936). The readings were taken by a Pulfrich photometer, using the green filter S. 53 and the violet filter S. 43. Double readings were in good accord, the average difference being 0.04 ± 0.016 mg %.

The packed cell volume was estimated by means of van Allen haematocrite. The samples were centrifugalized for 30 minutes. Double readings showed good accordance (average difference of 0.4 ± 0.19 %).

For the measurement of the red cell diameter smears of blood were stained by the May-Grünwald-Giemsa method. The measurement was performed after the method of Hynes and Martin (1936), 500 cells for each sample were measured. As, however, supravital staining might be assumed to damage the cells and thus alter their shape, the results were controlled in a minor part of the experiments by measuring the diameters of 200 red cells suspended in physiological saline. The mean erythrocyte diameter was calculated after Price-Jones (see Whitby and Britton, 1950, p. 648). The average thickness of the red cells was estimated on the basis of the mean diameter, the packed cell volume and erythrocyte count, from the formula $d = \frac{40}{\pi} \cdot \frac{H}{Er \cdot D^2}$ (d = mean thickness, H = packed cell volume, Er = million of erythrocytes in cu.mm, D = mean diameter).

Local venous anoxaemia was induced in the test subjects by obstructing the venous circulation in one limb. For this purpose the tourniquet of the Riva-Rocci blood pressure apparatus was wound round the arm of adult test subjects and of older children, or round the thigh of young children, and a pressure of 40—60 mm Hg was led into the tourniquet and maintained for one hour. In the experimental group comprising the children the number of erythrocytes, the haemoglobin and the percentage of reticulocytes was determined immediately before the application of the pressure, and on 2 or 3 successive days, at the same hour. In the first group of adults comprising 15 subjects, before the obstruction, haemoglobin, erythrocytes and leucocytes were determined from the capillary blood, and samples of venous blood were obtained for determination of the serum bilirubin, mean erythrocyte diameter and packed cell volume. Immediately before the letting out of the pressure the number of leucocytes, and, in some cases, the number of erythrocytes and haemoglobin, were estimated from the capillary blood of the anoxic limb, and the serum bilirubin, the mean erythrocyte diameter and the packed cell volume were determined from a sample of venous blood obtained from the anoxic limb. After the treatment, erythrocyte and haemoglobin values were determined on a few successive days. In the second group of adults, consisting of 10 subjects, only haemoglobin and erythrocytes were determined before the treatment and on a few days following. In 5 cases erythrocyte and haemoglobin values were determined also 10—15 minutes after the setting on of the tourniquet. Samples of venous blood for dertermination of oxygen were taken before the obstruction and immediately before the pressure was released.

The whole experimental data was subjected to statistical treatment. For calculations of the mean error of a single determination (δ) and the mean error of the mean (ε) Fechners formulae $\delta = \pm \frac{1.25\ \Sigma \Delta\chi}{n-0.2}$ and $\varepsilon = \pm \frac{1.25\ \Sigma \Delta\chi}{(n-0.2)\sqrt{n}}$ were used ($\Delta\chi$ = the deviation of a single determination from the mean, n = the number of determinations). Fechners formulae,

though more simple, have been shown to yield values for δ and ε consistent to those obtained by the generally used, more complicated formulae $\delta = \pm \sqrt{\frac{\Sigma \Delta\chi^2}{n-1}}$, $\varepsilon = \pm \sqrt{\frac{\Sigma \Delta\chi^2}{n\,(n-1)}}$ (I. Bonsdorff 1943), when applied to experimental series of a normal distribution. The validity of Fechners formulae for the present data has been confirmed by calculating, in a part of the data, δ and ε after the formulae $\delta = \pm \sqrt{\frac{\Sigma \Delta\chi^2}{n-1}}$, $\varepsilon = \pm \sqrt{\frac{\Sigma \Delta\chi^2}{n\,(n-1)}}$ as well. It was then found that both formulae gave consistent results. The present data was also shown to be in accordance with the normal distribution curve of Gauss. The coefficient t for statistical significance of the difference of two series with the means M_1 and M_2 was calculated after the formula $t = \frac{M_1 \div M_2}{\varepsilon_{M_1 \div M_2}}$, where $\varepsilon_{M_1 \div M_2}$ is the mean error of the difference, $\varepsilon_{M_1 \div M_2} = \pm \sqrt{\frac{\Sigma \Delta\chi_1^2 - \Sigma \Delta\chi_2^2}{n_1 + n_2 - 2}} \cdot \sqrt{\frac{n_1 + n_2}{n_1\, n_2}}$. When t and $n_1 + n_2 - 2$ are known, the probability P is obtained from Fischer's tables.

RESULTS

Group 1. Effect of Local Venous Obstruction on the Peripheral Blood Picture. — To test the effect of local venous obstruction of a short duration on peripheral blood, the red cell count, the haemoglobin value and the percentage of reticulocytes on the first days following the obstruction was compared to those observed immediately before the treatment in a group of 30 children (Table I_1).

The results of these experiments are given in Fig. I_1 and in Table I_2. As will be seen from the table all the test subjects with the exception of a few reacted to the treatment with an increase in the red cell, haemoglobin and reticulocyte values. The red cell count usually reached its maximum on the 1st or 2nd day after the treatment, and was levelled again on the 3rd or 4th day. The values for haemoglobin and reticulocytes, on the other hand, remained at a higher level throughout the experimental period. The increase in haemoglobin was less pronounced as compared to that of the red cells, and the colour index was correspondingly lowered. The reticulocytosis, even as present in the largest number of the cases, was slight as a rule, and never of a magnitude comparable to the reticulocyte peak seen for instance in pernicious anaemia under liver treatment. A typical single reaction is shown in Fig. I_1.

TABLE I_1

AGE, DIAGNOSIS AND BLOOD COUNTS OF CHILDREN TREATED WITH LOCAL VENOUS OBSTRUCTION

Test Subject	Age	Diagnosis	Blood Count		
			Er	Hb	R%
S.K.	3 years	Debilitas	3.8	46	1.3
M.G.	11 »	Convalescentia post malariam	4.3	72	1.2
E.A.	7 »	Urethra duplex. Enuresis	4.3	81	0.6
E.V.	2 »	Enuresis nocturna	4.7	73	0.4
L.H.	8 »	Diabetes mellitus	4.2	76	0.5
L.K.	13 »	Diabetes mellitus	5.8	96	0.4
L.M.	5 »	Tumor cerebri	3.9	86	0.8
H.S.	2 »	Deformitas congenita costae et columnae vertebralis	3.4	68	1.1
R.L.	8 »	Coxa plana l.a.	3.6	73	1.6
S.T.	10 »	Diabetes mellitus. Epilepsia	4.9	76	1.2
M.S.	10 »	Convalescentia post choream minorem	3.9	68	1.4
A.G.	3 »	Convalescentia post pyelocystitidem	3.3	76	1.0
B.I.	3 »	Enuresis diurna et nocturna	3.9	72	2.0
T.L.	13 »	Dystrophia adiposo-genitalis	4.4	82	1.3
L.A.	7 »	Enuresis diurna	4.0	74	0.8
S.L.	11 »	Diabetes mellitus	4.1	82	1.4
P.J.	4 »	Enuresis nocturna	4.7	82	1.2
K.R.	6 »	Diabetes mellitus	3.9	80	1.0
J.L.	3 »	Convalescentia post encephalitidem	3.9	66	0.6
S.K.	3 »	Debilitas	3.7	65	1.2
H.H.	11 »	Diabetes mellitus	4.3	80	1.0
R.K.	7 »	Nihil obs.	4.6	80	—
T.N.	14 »	Condrodystrophia feotalis	3.4	74	—
T.P.	13 »	Diabetes mellitus	4.1	88	—
G.L.	6 »	Diabetes mellitus	3.4	84	—
R.M.	9 »	Enuresis nocturna. Incontinentia ani	4.8	77	1.1
E.K.	12 »	Enuresis nocturna	3.9	82	1.2
S.T.	9 »	Enuresis	4.5	75	1.5
P.M.	10 »	Enuresis nocturna. Inocentinentia ani	4.1	80	2.0
E.A.	4 months	Nihil obs.	3.9	69	1.4

TABLE 1.

EFFECT OF VENOUS OBSTRUCTION IN A LIMB ON THE PERIPHERAL ER COUNT, HB AND R % OF CHILDREN

Test Subject	Erythrocytes Mill./cu.mm				Haemoglobin				Reticulocyte %			
	Initial Value	Deviation from Initial Value			Initial Value	Deviation from Initial Value			Initial Value	Deviation from Initial Value		
		1st Day	2nd Day	3rd—4th Day		1st Day	2nd Day	3rd—4th Day		1st Day	2nd Day	3rd—4th Day
S.K.	3.8	—	+1.2	—0.2	46	—	0	+ 2	1.3	—	+0.9	+1.4
M.G.	4.3	—	+0.3	—0.3	72	—	+ 2	+ 8	1.2	—	+1.4	+3.7
E.A.	4.3	+0.3	+0.3	0.0	81	+ 5	+ 5	— 1	0.6	—0.1	+0.3	+1.6
E.V.	4.7	+0.1	+0.3	—	73	+ 7	— 1	—	0.4	+1.2	+0.4	—
L.H.	4.2	+0.4	+0.5	—	76	+ 1	+ 4	—	0.5	0.0	+0.5	—
L.K.	5.8	0.0	+0.3	—	96	+ 4	— 5	—	0.4	0.0	+0.6	—
L.M.	3.9	+0.3	+0.4	+0.2	86	0	— 6	0	0.8	+0.4	+0.2	+1.0
H.S.	3.4	+0.4	+0.4	—	68	0	+12	—	1.1	+0.3	+1.3	—
R.L.	3.6	+0.5	—0.1	+0.2	73	— 1	— 7	— 3	1.6	—0.6	+0.2	+0.8
S.T.	4.9	—0.3	—0.6	—0.5	76	0	+ 2	0	1.2	+0.4	+2.6	+1.0
M.S.	3.9	+0.5	+0.4	—0.2	68	0	+10	+14	1.4	0.0	+1.0	+0.8
A.G.	3.3	+0.5	+0.3	—	76	0	0	—	1.0	+0.4	+1.0	—
B.I.	3.9	+0.5	+0.5	+0.4	72	+ 2	+ 2	+ 2	2.0	+0.1	+0.2	—0.4
T.L.	4.4	0.0	0.0	0.0	82	+ 8	+ 4	0	1.3	+0.1	+0.1	—0.1
L.A.	4.0	—0.1	+0.2	0.0	74	0	+ 2	0	0.8	+0.1	+0.2	—
S.L.	4.1	0.0	0.0	0.0	82	0	— 2	+ 2	1.4	+0.6	—0.1	—
P.J.	4.7	+0.1	+0.1	0.0	82	— 7	+ 6	+ 2	1.2	—0.2	—0.2	+0.2
K.R.	3.9	+0.4	+0.2	0.0	80	+ 7	0	0	1.0	+1.1	+0.8	+1.2
J.L.	3.9	—	+0.7	0.0	66	—	+16	+11	0.6	—	+0.8	+1.0
S.K.	3.7	+0.6	+1.0	—	65	+10	+ 9	—	1.2	+0.4	+0.4	—
H.H.	4.3	+0.4	+0.2	+0.1	80	+10	+ 4	— 2	1.0	+0.8	+1.0	+0.8
R.K.	4.6	+0.2	—0.1	—	80	+ 4	— 2	—	—	—	—	—
T.N.	3.4	0.0	+0.6	—	74	+ 6	+ 6	—	—	—	—	—
T.P.	4.1	+0.2	—	0.0	88	0	—	— 4	—	—	—	—
G.L.	3.4	+0.9	—	+1.1	84	0	—	— 2	—	—	—	—
R.M.	4.8	—	0.0	—0.1	77	—	+ 1	+ 9	1.1	—	+1.9	+0.1
E.K.	3.9	—	+0.5	+0.4	82	—	+ 2	+ 1	1.2	—	+0.4	—0.4
S.T.	4.5	—	+0.3	+0.3	75	—	0	+ 7	1.5	—	+0.5	—0.4
P.M.	4.1	—	+0.4	+0.3	80	—	+ 1	0	2.0	—	+2.0	—0.9
E.A.	3.9	+0.7	—	+0.4	69	0	—	0	1.4	0.0	—	—0.1
Mean	4.12 ± 0.10	+0.29 ±0.06	+0.31 ±0.06	+0.10 ±0.06	76.1 ± 1.4	+ 2.4 ± 0.9	+ 2.4 ± 0.9	2.1 ± 0.9	1.12 ±0.08	+0.26 ±0.09	+0.73 ±0.13	+0.63 ±0.23
n	30	23	27	22	30	23	27	22	26	19	25	18

Statistical treatment of the data shows the increase in the red cell count to be highly significant on the 1st and 2nd day after the treatment, while on the following days the deviation from the initial value cannot be statistically confirmed. The increase in haemoglobin is statistically probable on the two first as well as on the later days. As regards the reticulocyte reaction, the increase as

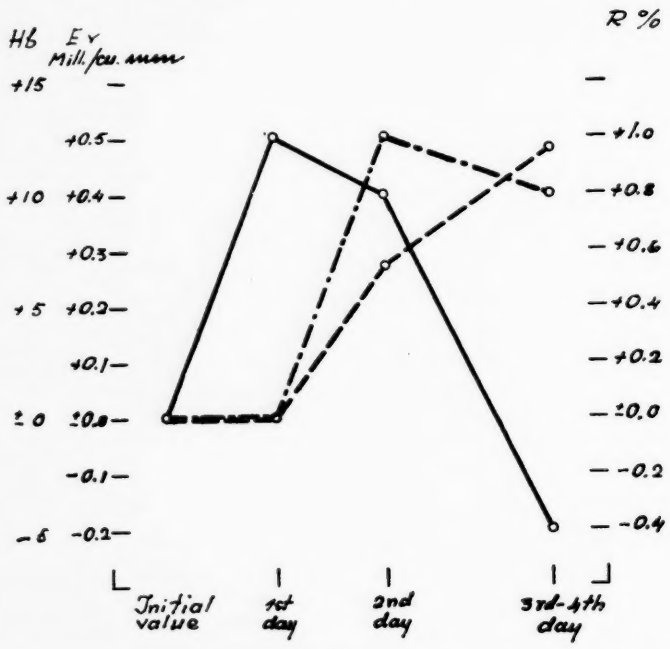

Fig. I_1. Effect of venous obstruction on the erythrocyte count, the haemoglobin value and the percentage of reticulocytes of child M.S. Er o———o, Hb o— — — o, R o— · — · —o.

compared to the initial value is well confirmed statistically on the 2nd day, and the average increase on the 1st and 3rd—4th days also is highly probable.

When considering Table I_2 it will be easily seen that the greatest single increases are mostly encountered in subjects with initially low erythrocyte values. This is more clearly demonstrated if the data are divided into two parts, the first group comprising those subjects with Er $\leqq$ 3.9 Mill./cu. mm, the second those with Er $\geqq$ 4.0 Mill./cu.mm. The average reaction of the red cells is very

marked in the former group, but considerably slighter in the latter. If the average deviation from the initial value in both groups on the two first days are compared, the difference will be found statistically significant, the coefficient t for the first day being 4.1 ($P < 0.001$) and for the second day 3.0 ($P < 0.01$).

To summarize, the present data indicate that obstruction of the venous circulation for a period of one hour is followed by a marked increase in the red blood cells, as well as by a moderate increase in the haemoglobin value and in the percentage of the reticulocytes. This reaction is more pronounced if the red cell value of the test subject is low.

Group 2. Effect of Local Venous Obstruction on the Oxygen Saturation and the Oxygen Capacity of Venous Blood. — The experiments reported above indicate the erythrocytosis promoting effect of local venous anoxaemia. The second experimental series was devised in order to gain some knowledge of the changes of blood in stagnant anoxia which might be held responsible for the reaction. The first factor to be considered is the reduced oxygen content of stagnant blood. For this purpose the oxygen capacity and the percentage of oxygen saturation of the venous blood before and at the end of the venous obstruction were measured in 10 healthy adult test subjects. The values for the oxygen capacity varied from 14.9 to 21.0 cc 0_2 per 100 cc of blood and for the percentage of oxygen saturation from 52.3 to 95.2 before the obstruction.

The effect of venous obstruction on the peripheral blood picture was controlled by enumerating the red cells and determining the haemoglobin value before the obstruction and after an interval of 1—5 days from the treatment. The results are given in Table I_3. It will be noted that the increase in the erythrocytes, though slight, is yet statistically significant, and the increase in haemoglobin is well within the limits of statistical probability.

The values for the oxygen saturation and oxygen capacity before and at the end of the application of the tourniquet, and the erythrocyte count before and during the application of tourniquet are summarized in Table I_4. The last column, marked »Reaction», indicates the effect of the treatment on the subjects' erythrocyte count on the following days (see Table I_3). Those cases in which the increase in the erythrocyte count amounted to or over 0.3 Mill./cu.mm are marked with +, those with an increase less than 0.3 Mill./cu.mm with —.

TABLE I_3

EFFECT OF VENOUS OBSTRUCTION IN ONE ARM ON THE RED CELL COUNT AND HAEMOGLOBIN OF ADULTS

Test Subject	Erythrocytes Mill./cu.mm			Haemoglobin		
	Initial Value	Deviation from Initial Value		Initial Value	Deviation from Initial Value	
		1st Day	2—5 Day		1st Day	2—5 Day
Y.H.	4.6	—	+0.4	86	—	— 1
S.L.	3.9	+0.1	+0.7	65	+11	+13
Y.H.	5.1	+0.2	+0.3	85	— 3	+ 1
B.Ö.	4.2	—	+0.2	93	—	+ 2
A.K.	4.7	—	+0.3	87	—	+ 8
L.P.	4.3	—	—0.3	85	—	0
H.L.	4.2	+0.3	+0.2	91	+ 1	0
V.M.	4.2	—	+0.1	80	—	+ 7
I.K.	4.9	—	+0.1	99	—	+10
H.P.	3.7	—	+0.3	80	—	+ 5
Mean	4.38 ±0.14	+0.20	+0.23 ±0.07	85.1 ± 2.4	+ 3.0	+4.5 ±1.6
n	10	3	10	10	3	10

TABLE I_4

EFFECT OF VENOUS OBSTRUCTION LASTING 1 HOUR ON THE VENOUS PERCENTAGE OF OXYGEN SATURATION, ON THE VENOUS OXYGEN CAPACITY AND ON THE ER COUNT IN CAPILLARY BLOOD

Test Subject	% of O_2 Saturation		O_2 Capacity Vol. %		Er in Capillary Blood		Reaction
	before	after	before	after	before	after 10 minutes	
Y.H.	86.0	36.5	18.5	18.9	—	—	+
S.L.	52.3	20.5	19.5	19.5	—	—	+
Y.H.	95.2	75.0	19.2	22.6	—	—	+
B.Ö.	85.0	73.1	16.2	18.3	—	—	—
A.K.	81.8	42.0	14.9	20.5	—	—	+
L.P.	79.7	49.5	19.0	19.6	4.3	4.3	—
H.L.	56.0	45.0	21.0	22.2	4.2	4.7	+
V.M.	60.0	27.4	19.9	20.5	4.2	4.3	—
I.K.	58.0	57.5	20.7	21.1	4.8	4.8	—
H.P.	70.5	41.5	16.9	19.2	3.7	3.8	+
Mean	72.5 ± 4.9	46.8 ± 4.2	18.6 ±0.62	20.2 ±0.45	4.24	4.38	
n	10	10	10	10	5	5	

As seen from the table, the percentage of oxygen saturation of the venous blood suffered, as a rule, a marked decrease during the circulatory obstruction. In one case only (I.K.) there was practically no change in the oxygen saturation.

As regards the oxygen capacity, a general increase after the obstruction was noted, due probably to a slight haemoconcentration. On the other hand, the erythrocyte count performed using capillary blood of the obstructed hand 10 minutes from the beginning of the obstruction showed only in one case (H.L.) an increase of magnitude similar to the erythrocyte reaction on the days following the treatment, while in other subjects there was no change in the number of red cells.

When comparing the average values for the oxygen saturation before and after obstruction (Table I_4 bottom line) it is seen that the difference between the two series is statistically significant ($t = 3.5$, $P < 0.01$). As regards the oxygen capacity the difference between the average values before and after obstruction cannot be statistically confirmed ($t = 2.1$, $P = 0.05$), probably because the test subjects were too few.

If the oxygen saturation after obstruction is considered in correlation to the subsequent erythrocyte reaction (columns 3 and 8), it is seen that a low value of oxygen saturation after obstruction often coincides with a positive erythrocyte reaction, while in cases with high oxygen saturation after obstruction the reaction is often absent.

To summarize: According to the present experiments circulatory obstruction in one arm, lasting one hour, causes a slight, yet statistically significant increase in the red cell count of adult subjects. During stagnation the percentage of oxygen saturation of venous blood decreases significantly. There is also a slight increase in the oxygen capacity of the blood during obstruction, which falls within the limits of statistical probability.

Group 3. Effect of Local Venous Obstruction on the Serum Bilirubin, the Red Cell Shape and the Packed Cell Volume of Venous Blood. — The destruction products of red cells have been claimed to have an erythrocytosis promoting effect (see Introduction). On the other hand, circulatory obstruction represents a condition which might be expected to increase the erythrocyte fragility, and, con-

sequently, an increased amount of destruction products of red cells in the blood might be expected. Recknagel (1930) claims the serum bilirubin to be increased by 50% during stagnation (pressure 100 mm Hg). Tsai, Chen and Chiu (1943) report increased erythrocyte fragility in stagnant blood while Mettier, Weaver and McBride (1949) observed no change in the osmotic resistence of erythrocytes in stagnant blood from varicose veins as compared to cubital vein blood.

The following experiments were devised in order to decide, whether increased haemolysis and the thereby liberated or formed substances might be held responsible for erythrocytosis following circulatory obstruction.

The possible increase in the destruction products of haemoglobin was controlled by determining the serum bilirubin content in the venous blood before and after obstruction. As another criterium for the tendency for haemolysis the shape changes of the red cells were considered. According to the generally accepted theory, the final haemolysis of red cells (in hypotonic solutions) is preceded by a change of shape towards the spherocyte type, which allows the volume of the cell to increase to a certain degree without affecting the ultra structure of the cell surface, see Ponder 1948 (p. 101). As an appropriate measure for the shape of the red cell the ratio thickness/diameter was calculated on the basis of the mean red cell diameter, of the number of red cells per cu.mm and of the packed cell volume (see Methods).

A leucocyte count from the capillary blood was performed before and after obstruction. This was done because it could be expected that if the treatment caused some deep disturbance in the congested area, this might probably mirror in the leucocyte count, causing increased immigration of white cells either into the blood of the congested limb, or from the blood into the tissues.

15 healthy adults of both sexes served as test subjects.

The effect of obstruction was controlled in this group, as before, by enumerating the red blood cells and determining the haemoglobin before the treatment and on a few succeeding days. In most cases there was an increase in the red cell count, the maximum being reached usually on the 3rd or 4th day. The elevation of the haemoglobin level was, as a rule, less pronounced than the erythrocyte reaction, and accordingly the colour index was decreased. The

TABLE I_5

EFFECT OF VENOUS OBSTRUCTION IN ONE ARM ON THE PERIPHERAL RED CELL COUNTS AND HAEMOGLOBIN VALUES OF ADULTS

Test Subject	Erythrocytes						Haemoglobin					
	Initial Value	Deviation from Initial Value					Initial Value	Deviation from Initial Value				
		1st Day	2nd Day	3rd Day	4th Day	5th Day		1st Day	2nd Day	3rd Day	4th Day	5th Day
E.B.	3.9	+0.1	+0.3	—	—	—	71	0	+ 7	—	—	—
O.E.	4.9	0.0	—0.6	—0.4	—	—	76	0	+19	+11	—	—
L.A.	5.4	—0.1	+1.0	0.0	—	—	79	0	+10	+ 3	—	—
L.T.	3.8	+0.2	+0.6	—	+1.2	—	80	+ 2	+ 2	—	0	—
J.L.	4.4	—	+0.2	—	+0.2	+0.4	75	—	+ 3	—	0	+ 5
T.M.	4.0	—	—0.3	+0.6	+0.6	+1.2	72	—	+ 1	+ 2	+ 2	+ 4
A.M.	4.6	0.0	+0.2	+0.4	—	—0.1	73	0	0	— 1	—	— 3
A.E.	4.3	+0.2	—	—	+0.5	+0.2	72	+ 1	—	—	+ 6	0
H.Å.	4.1	+0.2	—	+0.3	—	+0.8	80	0	—	+ 1	—	+ 2
S.U.	4.7	—0.8	—0.7	—0.6	+0.9	—	76	0	0	0	—1	—
H.Å.	4.1	—	+0.2	+1.1	—	0.0	73	—	+ 3	+ 3	—	+ 3
J.R.	3.7	+1.3	—	+0.7	—	+0.7	80	+13	—	0	—	— 1
T.L.	3.9	—	+0.6	+0.2	+0.2	+0.3	69	—	+ 1	0	0	0
U.B.	4.4	—0.6	—	+0.1	—	0.0	72	— 2	—	— 2	—	+ 2
K.K.	4.1	+1.2	—	+0.3	—	+0.4	72	+ 3	—	+ 3	—	+10
Mean	4.29 ±0.12	+0.16 ±0.16	+0.15 ±0.17	+0.25 ±0.15	+0.60 ±0.15	+0.39 ±0.13	74.7 ± 1.0	+ 1.5 ± 0.9	+ 4.6 ± 1.8	+ 1.8 ± 0.9	+1.2 ±1.0	+ 2.2 ± 1.1
n	15	11	10	11	6	10	15	11	10	11	6	10

TABLE

EFFECT OF VENOUS OBSTRUCTION IN ONE ARM ON THE SERUM BILIRUBIN, PACKED AND THICKNESS/DIAMETER RATIO OF VENOUS BLOOD,

Test Subject	Serum Bilirubin (mg %)		Packed Cell Volume %		Mean Diameter (μ)	
	before Treatm.	after Treatm.	before Treatm.	after Treatm.	before Treatment	after Treatment
E.B.	—	—	42	35	7.734 ±0.0218	7.638 ±0.0228
O.E.	—	—	—	—	7.834 ±0.0278	7.728 ±0.0262
L.A.	—	—	—	—	7.779 ±0.0251	7.896 ±0.0299
L.T.	0.67	0.26	45	42	7.820 ±0.0220	7.988 ±0.0204
J.L.	0.20	0.31	41	—	7.597 ±0.0205	7.836 ±0.0238
T.M.	0.32	0.01	36	—	7.837 ±0.0187	7.799 ±0.0222
A.M.	0.59	0.40	42	42	7.771 ±0.0193	7.829 ±0.0183
A.E.	0.36	0.24	40	40	7.828 ±0.0188	7.992 ±0.0183
H.Å.	0.31	0.36	38	45	8.101 ±0.0215	7.899 ±0.0205
S.U.	—	—	40	40	7.871 ±0.0216	7.965 ±0.0196
H.Å.	0.55	0.44	40	39	8.097 ±0.0204	8.200 ±0.0194
J.R.	0.30	0.29	37	37	7.383 ±0.0260	7.655 ±0.0220
T.L.	0.31	0.23	37	37	7.961 ±0.0210	7.913 ±0.0185
U.B.	0.58	0.61	42	44	7.935 ±0.0209	7.777 ±0.0205
K.K.	0.36	0.28	40	37	7.826 ±0.0201	7.900 ±0.0192
Mean	0.41 ±0.051	0.31 ±0.039	40.0 ± 0.65	39.8 ± 0.98	7.825 ±0.0221	7.868 ±0.0370
n	11	11	13	11	15	15

changes in the blood counts called forth by the obstruction are summarized in table I_5.

The variability of the red cell reaction in the present test subjects appears to be rather great and, as the number of the test subjects is not very large, the increase of erythrocytes is statistically confirmed only on the 4th day, and borders on significance on the 5th day. The range of variations in the haemoglobin reaction is also rather large, yet the average increase borders on statistical probability on 2nd—5th day.

Closer observation of the erythrocyte changes after obstruction suggest here, as in Table I_2, comprising the erythrocyte changes in children after local obstruction, that slightly anaemic test subjects react to the treatment with a more marked increase of erythrocyte number than those with normal initial erythrocyte

I_6

CELL VOLUME, MEAN ERYTHROCYTE DIAMETER, MEAN ERYTHROCYTE THICKNESS AND ON THE LEUCOCYTE COUNT IN THE CAPILLARY BLOOD

Mean Thickness (μ)		Thickness/Diameter Ratio		Leucocytes 1,000/cu.mm		Reaction
before Treatm.	after Treatm.	before Treatm.	after Treatm,	before Treatm.	after Treatm.	
2.93	1.98	0.250	0.260	5.6	10.2	+
—	—	—	—	6.4	4.8	—
—	—	—	—	4.0	3.4	+
2.51	2.22	0.322	0.278	5.6	5.3	+
2.05	—	0.270	—	9.0	7.2	+
1.87	—	0.239	—	11.0	8.8	+
1.92	1.89	0.247	0.242	5.2	5.0	+
2.11	2.02	0.270	0.253	4.8	5.8	+
2.10	2.02	0.259	0.256	11.8	8.2	+
1.81	2.06	0.230	0.258	8.2	6.0	+
1.90	1.84	0.235	0.224	5.8	7.4	+
2.47	2.26	0.355	0.295	8.4	7.6	+
1.92	1.94	0.241	0.245	6.4	5.8	+
1.67	1.77	0.210	0.228	7.6	9.2	—
2.11	2.16	0.270	0.273	6.8	5.4	+
2.03 ±0.064	2.01 ±0.046	0.261 ±0.0085	0.256 ±0.0056	7.11 ± 0.56	6.71 ± 0.54	
13	11	13	11	15	15	

values. This observation becomes more evident if the data is divided into two groups, one comprising the cases of initial erythrocyte value $\leqq 4.2$, the other those of initial value $\geqq 4.3$. The difference between the deviations from the initial value on the three first days in the two groups is striking, though the material is not sufficient to allow statistical treatment.

Table I_6 represents a comparison of the values for serum bilirubin, packed cell volume, mean erythrocyte diameter, mean erythrocyte thickness, thickness/diameter ratio, and leucocyte count before and after obstruction. In the last column the reaction of erythrocytes is given: those cases in which the highest deviation from the initial value amounts to or exceeds 0.3 Mill./cu.mm are marked with +, while those with the highest increase remaining below 0.3 Mill./cu.mm are recorded as negative cases and marked with —.

The serum bilirubin values after obstruction show, in individual cases, minor deviations in both directions as compared to the values before obstruction. In two cases (L.T. and T.M.), however, there is a marked decrease in the bilirubin values. These great deviations are mainly responsible for the difference in the average values (bottom line), which, though not significant, yet may attract the readers attention.

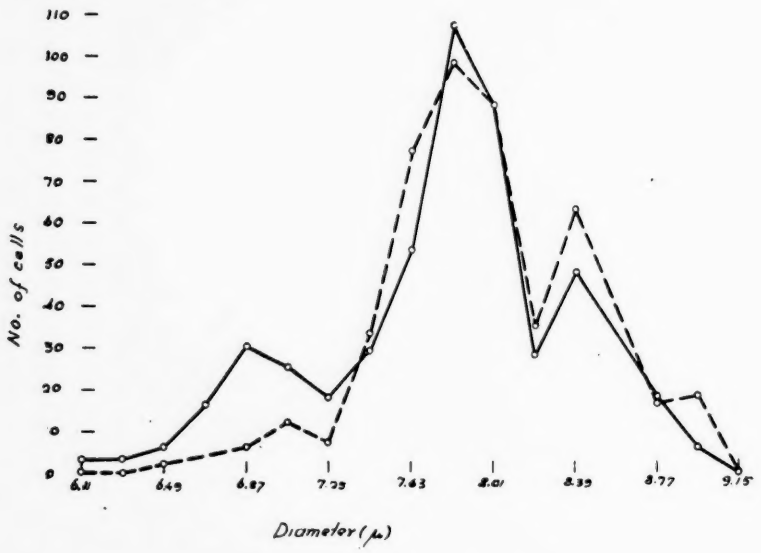

Fig. I_2. Price-Jones curves of subject L.. T. before and after venous obstruction. Before c———o, after o— — —o.

The packed cell volume in individual test subjects showed rather marked deviations in both directions. There is, however, no difference between the average values before and after congestion. This result indicates that the circulatory obstruction did not cause any consistent haemoconcentration.

In the mean erythrocyte diameter only negligible increases and decreases were encountered after the obstruction, and the average value given on the bottom line differs little from the average before obstruction. The individual Price-Jones curves showed no deviations from the normal shape either before or after obstruction. (Fig. I_2 shows the Price-Jones curves before and after treatment

of subject L.T.) As indicated by the similarity of the mean errors of mean erythrocyte diameter in individual test subjects before and after obstruction — given after the mean diameters — the congestion did not cause any anisocytosis.

As might be expected from the constancy of packed cell volume and mean erythrocyte diameter, the mean erythrocyte thickness remains nearly unchanged as well in individual cases as in the average. The same holds good for the ratio thickness/diameter (columns 10—11, bottom line). Accordingly, there is no change either in the volume or in the shape of the red cells during congestion.

The deviation of the leucocyte count from the beginning to the end of congestion is fairly marked in some individual cases, yet the values hardly ever fall outside the range considered normal for a healthy person (4,000—11,000/cu.mm, see Whitby and Britton 1950, p. 31). This inconstancy might be interpreted to show that during stagnation, immigration of leucocytes into the blood of the congested area or from the blood into the tissues has occurred. As deviations in the leucocyte number, however, are not observed in all cases, the local venous obstruction can hardly be considered to have caused a serious disturbance in the congested area which should be considered when interpreting the results of the experiments.

To summarize the results described above, it is shown that circulatory obstruction in an arm for one hour promotes erythrocytosis in adult persons. Test subjects with low erythrocyte values react more strongly than those with a normal erythrocyte count. The amount of bilirubin in serum at the end of the congestion is slightly lower than at the beginning, this, however, may be attributed to a marked decrease of bilirubin in two cases, rather than to a general tendency to decrease. The volume and shape of red cells do not suffer any change during congestion; there is no sign of sphaerocytosis. — Thus, there is no evidence of increased red cell destruction during congestion, nor of any change in the erythrocyte shape towards the prae-haemolysis type.

DISCUSSION OF PART I

The present experiments suggest that induced venous obstruction of short duration in a limb brings about a statistically con-

firmed erythrocytosis in the peripheral blood which was observed on a few days following the treatment. Examination of the venous blood at the end of the obstruction showed a significant decrease in the oxygen saturation. No signs of increased red cell destruction, on the other hand, were observed.

When attempting to interpret this result, two questions seem to be of the greatest interest: 1. the nature of the erythrocytosis and 2. the stimulation mechanism.

As to the nature of the erythrocytosis, the same possibilities as in regard of the high mountain erythrocytosis — see Introduction — must be considered. Haemoconcentration might suggest itself because of the signs of haemoconcentration which are observed during the obstruction in group 2, e.g. increased oxygen capacity and increased red cell count 10 minutes after the onset of the obstruction. Haemoconcentration during local circulatory obstruction has also been observed by earlier authors (Recknagel, Mettier, Weaver and MacBride). Closer consideration, however, makes haemoconcentration as the cause of the erythrocytosis in the present material less likely. Local haemoconcentration in the obstructed arm could not account for erythrocytosis observed similarly in blood from both hands. General haemoconcentration, on the other hand, could hardly be expected after a procedure as innocuous as venous obstruction in a limb for 1 hour; and for haemoconcentration lasting 2—3 days a serious disturbance of the water balance of the body would be needed.

Thus, erythrocytosis due to haemoconcentration can be disregarded. The same holds good in regard of redistribution of the formed elements of the blood; though some redistribution might occur during and immediately after the obstruction, it cannot reasonably be expected to last for several days.

The emptying of the blood depots merits more serious consideration. It may be assumed that during circulatory obstruction some products of tissue destruction or some intermediates of metabolism might gather into the congested blood, and after the restoration of normal circulation, reach the blood depots and stimulate them to contraction. Nervous impulses from the obstructed area giving rise to reflectory contraction in the blood depots cannot, either, be excluded offhand. The relatively long duration of the erythrocytosis, however, seems contradictory to the depot contrac-

tion as the only cause of the peripheral erythrocytosis. Contraction of the spleen caused by adrenalin, work etc., is known to last only a few hours. Thus, a state of constant contraction of the blood depots would require continuous stimulation which is not the case in the present experimental conditions.

Increased release of red blood cells from the bone marrow remains, thus, the most likely explanation for the erythrocytosis. This theory is supported by the occurence of reticulocytosis, which was observed in children, and by the discrepancy of the red cell and haemoglobin values during the experimental period observed in all experimental groups. — Both these arguments, reticulocytosis and changes in the colour index have been emphasized also as a proof for the opinion that the high mountain erythrocytosis is due to increased release of erythrocytes from the bone marrow (see Introduction).

The most important objection, on the other hand, against interpreting the erythrocytosis in the present test subjects as due to bone marrow stimulation is the rapid onset, as well as the rapid disappearance, of the erythrocytosis. Maximal erythrocytosis in children was observed on the 2nd day, in adults on the 2nd — 5th day, reticulocytosis in children between 2nd and 4th day. On the 4th—6th day after the treatment the values were usually lowered again. As regards anoxic erythrocytosis (high mountain or simulated altitudes) the immediate rise of erythrocytes and haemoglobin is usually considered to be due to the emptying of the blood depots, while erythrocytosis based on increased erythropoietic activity is not expected until after a lag of 1—3 weeks (see Introduction). This conception, however, is purely hypothetical, because it is not based on examination of erythrocyte maturation in the bone marrow under anoxic conditions. A single bone marrow biopsy, carried out during anoxic erythrocytosis, only yields some information of the time relations of each stage of erythrocyte maturation, but not of the length of the whole process.

Besides direct bone marrow examination, the lag between the administration of a recognized erythropoietic stimulant and the reticulocytosis and erythrocytosis caused by it can be considered as a criterion of the maturation time of erythrocytes. According to Whitby and Britton (1950, p. 78) administration of active liver substance causes, in pernicious anaemia patients, a reticulocytosis,

which appears on the 2nd to 10th day after the onset of treatment, and reaches a maximum on the 3rd—10th day. Erythrocytosis is observed somewhat later than the reticulocyte peak. Jacobson and Williams (1945), working on splenectomized rabbits, arrive at very nearly the same time for the appearance of reticulocytosis, i.e. 2—6 days. Though these values indicate a maturation time rather longer than suggested by the present experimental data, they are still not totally incompatible. For, as Whitby and Britton point out, the erythrocyte and reticulocyte response is influenced by several factors, e.g. amount of the material administered, the method of administration, the rate of absorbtion, and the reactive power of the bone marrow. Furthermore, there is no certainty that the erythropoiesis stimulating mechanism in the present experiments attacks the same stage in erythrocyte maturation as the antipernicious principle, and thus the two cases may not be wholly comparable. — Another indication of the ripening time of erythrocytes, though probably no more comparable with the present data, is given by the statement of Hahn and co-workers (1940) that marked iron atoms are detected in the circulating erythrocytes of anaemic dogs a few hours after feeding. On the basis of theoretical calculus on the erythron, Karvonen (1948) arrives at maturation time from 23 hours to 17 days, a range, which is well compatible with the present data as well as with the results obtained as regards of liver treated pernicious anaemic patients.

The rapidly onsetting erythrocytosis in the present test subjects is, on the other hand, in good accord with the erythrocytosis which takes place after injection of anoxic haemopoietins. (See Verzár and co-workers, Döring, Klingelhöffer, Loeschke, Bonsdorff and Jalavisto.) This fact, however, cannot be held as a decisive proof of the present reaction being due to increased erythropoiesis, as the erythropoiesis stimulating actiyity of anaemic or low pressure plasma has not been controlled by bone marrow examination, save for the work of Krumdieck, which comprises only a few experiments. Yet, there is no reason to believe that, while anoxia itself stimulates erythropoiesis, anoxic haemopoietins would act on blood depots. The similarity between erythrocytosis brought about by haemopoietins and by local venous obstruction, supports the view that the present erythrocytosis is also of bone marrow origin.

The rapid disappearance of the erythrocyte and reticulocyte reaction is not difficult to explain after closer consideration. As the present test subjects were normal as regards their blood counts, and probably also their blood forming organs, the increase of erythrocytes by some 0.5 Mill./cu.mm would be unnecessary and thus disturbe the natural balance. As a result, a normal organism would strive to regain the balance by means of either increased erythrocyte destruction, or, preferably, by decreased erythrocyte formation. — In anaemic subjects, erythrocytosis following local venous obstruction is observed to last for a longer period (Bonsdorff and Seleste 1950), here the increase of oxygen carriers is needed, and therefore, possibly, the balancing mechanisms are not set to work actively. Furthermore, erythrocytosis after injection of anoxic haemopoietins in normal test animals disappears as quickly as in the present test subjects (Bonsdorff and Jalavisto).

The increase of erythrocytes and reticulocytes in the present test subjects is rather slight in average. This, however, is not very unexpected, as obstruction was induced only in a relatively small part of the circulation and lasted only one hour. Furthermore, reactions of considerable magnitude were observed in some subjects, while others showed no reaction; thus the average slightness of the reaction is rather due to these »negative» cases than to the insignificance of the reaction in the »positive» ones. — It may be noted that even in magnitude, the reaction of the present test subjects is very similar to that observed earlier in test rabbits after injection of anoxic haemopoietins (Bonsdorff and Jalavisto).

To summarize what has been said above, it is probable that the peripheral erythrocytosis after venous congestion in a limb is due to stimulation of the bone marrow.

Another major question is whether the stimulation of the bone marrow is local — in the congested limb — or general, transmitted by a humoral agent. Either direct examination of the bone marrow in the congested, resp. untreated limb, or injections of plasma from the congestion blood into test animals would be needed to solve this question. The former suggestion could not, however, be carried out on human subjects, and, as regards the latter, the haemolysing effect of human plasma when injected into rabbit (experiments to be published later) was a serious obstacle which could not be surmounted as yet. Thus, while leaving the question unsolved so far,

some speculation on the matter may be allowed. Following considerations suggest a humoral stimulation of the whole erythron: 1. There is no evidence, and, indeed, little probability that the oxygen saturation in the bone marrow of the anoxic limb were decreased. 2. Considerable data has been presented by previous authors (see Introduction) showing that reduced oxygen content in the bone marrow blood does not stimulate erythropoiesis. 3. If the bone marrow contained in the area of congestion is estimated for, say 1/20 of the whole marrow, then, in order to cause an increase of 0.5 Mill./cu.mm in the peripheral erythrocyte count within a couple of days, an extreme activity would be needed, if the erythrocytosis were due to local bone marrow reaction: the congested bone marrow should be in a state of activity 20 times stronger than when, under low atmospheric pressure, an increase of 0.5 Mill. Er/cu.mm is observed within 2—3 days. It does not seem likely that venous congestion for one hour would represent so strong a stimulus. 4. The similarity of the erythrocytosis in the present test subjects and after injection of anoxic haemopoietins favours a similar mechanism of stimulation in both cases.

The study of the changes occurring in the stagnant blood yielded but few positive facts. The rôle of destruction products seems to be well excluded; no bilirubinaemia and no apparent changes in the red cell shape after obstruction. (According to a recent review by Crosby 1952, the increase in the thickness/diameter ratio in stagnant blood is reversible, and thus does not imply a shortened life expectancy of such red cells.) The only marked change revealed by the present experiments is the lowered venous oxygen saturation. The probability that low oxygen pressure in the blood might give rise to the formation of some erythropoietic substance, will be discussed later, in connection with Part II.

The present experiments give no information whether *anoxaemia* as such, or *tissue anoxia* in the congested area may be the decisive factor in causing the erythrocytosis in the present experiments. Therefore, a secons series of experiments was added to the present study: experiments on blood in vitro.

PART II

FORMATION OF HAEMOPOIETIC SUBSTANCES IN BLOOD IN VITRO

The results of Part I suggest that erythropoiesis stimulating substances are formed during induced venous hypoxaemia in a limb. This observation encouraged the writer to investigate the formation of the active substances in further simplified conditions, in which the rôle of other tissues is excluded, e.g. in blood in vitro. For this purpose the erythropoietic effect of plasma from blood kept under low atmospheric pressure was compared to the effect of plasma from untreated blood. Though the results of these experiments seemed consistent (see Results), several later attempts of the writer, as well as others (Kinard and Ellis 1949) to reproduce the results have failed. Therefore, in order to decide, whether the erythropoietic effect of plasma subjected to low pressure might depend on the activity of the haemopoietic organ of the donor animal, the effect of plasma from blood subjected to low pressure containing high and low percentage of reticulocytes was compared.

TEST SUBJECTS AND METHODS

As test animals rabbits weighing 2—4 kg were used. As the reactivity of rabbits seems to vary in a remarkable degree (see Bonsdorff and Jalavisto), animals of the same breed were mostly used, and, when possible, litter-mates were chosen for the main experiments and for the corresponding controls. In a previous study it also appeared that during the warm season the rabbits hardly reacted to active haemopoietic substances; therefore the present experiments were performed between September and May.

The rabbits were kept in cages sufficiently large to allow some excercise, in a room of a relatively low temperature, +15° C on the average. A free supply of oats, swedes and spruce twigs, and of water made up the diet of the animals. Blood for determination of the number of erythrocytes, haemoglobin and the percentage of reticulocytes was obtained by pricking

the ear vein with a needle and letting a drop form. The samples were taken between 9 and 11 a.m. and the blood counts were performed directly afterwards. In the first part, comprising experiments on the presence of erythrocytosis promoting substances in blood subjected to low pressure in vitro, 0.2—0.5 cc of blood were collected into a heparinized syringe. From this sample the erythrocyte count and the determination of haemoglobin were performed as described in Part I. For the reticulocyte count a drop of fresh blood was placed on a dry slide dyed with brilliant cresyl blue and covered with a cover glass. In the latter part of the study, comprising the experiments on the formation of the erythrocytosis promoting factor in blood containing low and high percentage of reticulocytes, the blood for the erythrocyte count and haemoglobin determination was drawn into the pipette from a fresh drop of blood. The reticulocyte count in these experiments was performed from supravitally stained slides prepared as described in Part I, Methods. 1,000 red cells were counted for each reticulocyte determination.

The blood samples for the »low pressure» and »control» experiments were drawn from the heart of the rabbits, into a heparinized syringe, maximally 40 cc were taken each time. No special attention was given to whether the right or left side of the heart was punctured, but, according to the writer's estimation, venous and arterial blood were obtained with equal frequency. The anaemization of the donor rabbits in the last series of the experiments (high and low reticulocyte percentage) was also carried out by heart punctures, 20—30 cc were removed at a time.

Determinations of the oxygen content of the heart blood were performed by the constant volume method of van Slyke (Peters and van Slyke 1932). In some of the test subjects double determinations were performed, these showed a fair constancy, the average difference being 0.5 ± 0.12 vol%.

The erythrocyte count of the normal test rabbits varied from 4.4 to 6.8 Mill./cu.mm; the haemoglobin values ranged from 58 to 83 Sahli grades, and the percentage of reticulocytes from 2.4 to 7.2. The blood values of individual rabbits showed fairly large variations from day to day, fluctuations of more than 0.5 Mill. Er/cu.mm or more than 10 Sahli grades were, however, seldom encountered. A similar inconstancy of the rabbits' blood count has been previously observed during an earlier study (Bonsdorff and Jalavisto) and it can probably be attributed to some environmental factors, such as varying food and water intake, changes of temperature and light in the room, and to the general lability of the rabbits' reactions to different stimuli; emotional excitement is claimed to cause an increase in the packed cell volume of rabbit blood (Nice and Katz 1934). Some seasonal variation in the rabbits' red cell count and haemoglobin has also been observed. This, however, is of minor importance in the present test subjects, as the single experiments were always of short duration.

As will be noted, the red cell and haemoglobin values in the present test subjects are somewhat lower than those considered normal for rabbits. The percentage of reticulocytes in most rabbits lies at the upper limit of

the normal range (2—4%). No certain reason for this slight anaemia can be offered, though as possible factors the relative lack of light and of sufficient excercise may be suggested.

The experimental arrangement was as following: Blood samples were placed in test tubes into a low pressure chamber, and the pressure was lowered, by means of suction, gradually to 10—400 mm Hg. The samples remained under the low pressure for 1½—4 hours.

The normal control samples, obtained by the same punctures as the »low pressure samples» were left standing in the open air in uncovered test tubes for 1½—4 hours. Directly after removing the »low pressure» sample from the chamber, both the »normal» and the »low pressure» sample were centrifugalized, and plasma was injected intraperitoneally into normal test rabbits, 2.2—4.0 cc in each animal. This method of administration was chosen as the most simple and yet satisfactory: according to Courtice and Steinbeck (1950) heparinized plasma is absorbed rapidly from the peritoneal cavity of rabbits. The recipient rabbits tolerated the injection without any signs of disturbance. On the first day following the injection, however, the weight of some animals was decreased by some hundred grams, but always gained the normal level again on the second day.

As full blood was never injected, there appeared to be no need to consider the formation of specific antibodies. Therefore the same animals were used as recipients several times, though always at an interval of at least 5 days.

The red cell, haemoglobin and reticulocyte values of the test subjects were followed for 1—2 weeks, at intervals of 2—3 days, until a fair stability was reached. The last determinations, with few exceptions, were carried out on the day the injection was given. After the injection the red cells were enumerated and the haemoglobin determined on 4—5 successive days, and the reticulocyte count in the first group of test subjects was performed on the 2—3 first days.

In the last experimental group (»donors with high and low reticulocyte count») the percentage of reticulocytes from the donors capillary blood was determined directly before the heart puncture was performed.

Statistical treatment of the data was performed as described in Part I.

RESULTS

Control experiments. — As the experimental arrangement in the main series involves procedures, which might effect the test animals' blood count in an unexpected way (e.g. intraperitoneal plasma injections, daily removals of small amounts of blood, involving possibly emotional excitement), the results of the main experiments could not be evaluated except by comparison with control experiments performed on subjects similar to those of the main experiments.

TABLE

EFFECT OF INJECTION OF PLASMA FROM RABBIT BLOOD, SUBJECTED TO NORMAL PERCENTAGE

Recipient	Date	Erythrocytes				
		Initial Value	Deviation from Initial Value			
			1st Day	2nd Day	3rd Day	4th Day
111	15. 3. 48	5.5	+0.2	+0.2	—0.5	+0.1
144	22. 3. »	4.8	+0.1	—0.1	+0.1	—
A 20	5. 4. »	4.8	0.0	—0.1	0.0	—0.3
130	9. 4. »	4.7	0.0	+0.2	+0.3	0.0
150	29. 4. »	5.1	—0.5	+0.1	0.0	—0.3
88	9. 12. »	5.0	0.0	+0.1	—	—0.1
95	9. 12. »	5.3	—0.2	—0.2	—0.4	—
Mean		5.03 ±0.11	—0.06 ±0.08	+0.03 ±0.07	—0.08 ±0.11	—0.12 ±0.08
n		7	7	7	6	5

In an earlier study, intraperitoneal injections of normal rabbits' plasma has been shown to have no effect on the rabbits' blood picture (Bonsdorff and Jalavisto), but as the present test subjects might not be fully comparable to the earlier ones, another control series was considered necessary. For this purpose the blood samples drawn from the donor animals were, in some cases, divided into two parts, and, while one sample was exposed to lowered atmospheric pressure, the other was left standing in normal pressure for the same time ($1\,^1/_2$—4 hours). Plasma separated from the latter sample was injected intraperitoneally into 7 control rabbits, 2.5—4.0 cc in each. The injection never caused any striking changes in the recipients' red cell count, haemoglobin or reticulocytes, save small deviations in either direction, which usually remained within the normal range of daily variations. The results are summarized in Table II_1.

It is seen that the values for erythrocytes, haemoglobin and reticulocytes show a slight tendency to decrease; the average deviations from the initial values never, however, differ significantly from zero.

Thus, it can be concluded that the injection of homologous plasma from blood which has been subjected to normal atmospheric pressure for

II_1

PRESSURE, ON THE RED CELL COUNT, HAEMOGLOBIN AND RETICULOCYTE OF RABBIT

Haemoglobin					Reticulocyte %		
Initial Value	Deviation from Initial Value				Initial Value	Deviation from Initial Value	
	1st Day	2nd Day	3rd Day	4th Day		1st Day	2nd Day
58	+ 4	+4	+7	+11	3.4	+0.2	0.0
64	—14	—	—8	—	—	—	—
66	+ 2	—2	—2	— 6	4.0	—1.0	—1.0
74	+ 6	+4	+6	+ 6	7.2	+0.2	—0.2
78	0	+1	—8	— 7	4.4	—1.0	—
73	— 1	—2	—	—	5.8	+0.4	—1.0
78	+ 2	—1	—	—	4.9	—1.0	—2.6
70.1	— 0.1	+0.7	—1.0	— 1.0	4.38	—0.33	—0.96
± 3.0	± 2.0	±1.2	±3.7	± 4.6	±0.54	±0.39	±0.41
7	7	6	5	4	6	6	5

$1^1/_2$—4 hours, does not cause any change in the rabbits' red blood count beyond the range of normal daily variations.

Effect of plasma from blood subjected to low pressure on rabbits' red blood picture. — Samples of 2.2—4.0 cc of plasma separated from blood which had been exposed to lowered atmospheric pressure (10—400 mm Hg) were injected intraperitoneally into 18 normal rabbits. One of the recipients (No. 130) had previously been given injections of normal rabbit's plasma, and two served later as controls for the effect of normal rabbits' plasma (88 and 95). Two rabbits had repeated injections of the »low pressure plasma» (128 and 73) at intervals of 5 resp. 17 days, so that 20 injections were given altogether.

The injection of »low pressure plasma» caused, in most test subjects, on the 1st—3rd day, an increase in the red cell count amounting maximally to 0.9 Mill./cu.mm. On the 4th or 5th day the values were usually lowered again to the initial level or slightly under it. In some cases, there was no increase but a slight decrease in the number of red cells, similar to that encountered after injection of normal rabbit plasma. In haemoglobin values, deviations in both directions were noted after the injection, which usually remained

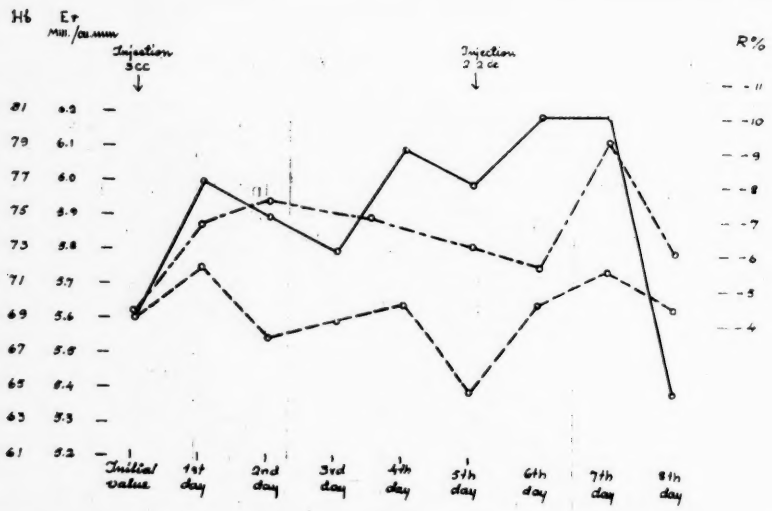

Fig. II_1. Effect of repeated injection of »low pressure plasma» on the erythrocyte count, the haemoglobin value and the reticulocyte percentage of rabbit 128. Er o———o, Hb o— — —o, R o—·—·—o.

within the limits of normal daily variation, but in some cases even greater deviations were observed (—18, +12). Some correlation between the erythrocyte and haemoglobin values was obvious, so that the greatest increases in erythrocyte number were usually accompanied by increased or unchanged haemoglobin values, and the greatest decreases in haemoglobin were observed in cases where there was no increase in the red cell number.

In the percentage of reticulocytes the injection of »low pressure plasma» caused a slight increase on the 1st—2nd day, which in most cases was levelled again on the 3rd—4th day. There appears to be no evident correlation between the change of erythrocyte and reticulocyte count after the injection.

A typical reaction of erythrocytes, haemoglobin and reticulocytes is shown in Fig. II_1 (rabbit No. 128, repeated injection of »low pressure plasma»).

In Table II_2 the results of these experiments are summarized.

Evaluation of the results indicates that the average increase of the erythrocyte count from the initial value is within the limits of statistical significance on the first two days after the injection, while the increase on the 3rd and 4th day cannot be statistically

TABLE II_2

EFFECT OF PLASMA FROM RABBIT BLOOD, SUBJECTED TO LOW PRESSURE, ON THE RED CELL COUNT, HAEMOGLOBIN AND PERCENTAGE OF RETICULOCYTES OF RABBIT

Recipient	Date	Erythrocytes					Haemoglobin					Reticylocyte %			
		Initial Value	Deviation from Initial Value				Initial Value	Deviation from Initial Value				Initial Value	Deviation from Initial Value		
			1st Day	2nd Day	3rd Day	4th Day		1st Day	2nd Day	3rd Day	4th Day		1st Day	2nd Day	3–4Day
A 26	15. 3. 48	5.2	+0.1	+0.3	+0.2	+0.5	63	+ 1	0	+12	+12	4.4	+0.4	—1.2	+2.8
A124	22. 3. »	4.7	+0.8	+0.9	—	—	61	+11	+ 3	—	—	—	—	—	—
A 50	5. 4. »	5.3	+0.5	+0.4	—0.3	—0.4	66	+ 3	+ 2	+ 1	+ 2	5.0	+2.6	+2.2	—0.8
148	» » »	5.0	+0.5	+0.5	+0.5	+0.2	68	0	— 3	+ 3	+ 4	2.4	+3.8	+3.5	+3.6
128	9. 4. »	5.6	+0.4	+0.3	+0.2	+0.5	69	+ 3	— 1	0	+ 1	4.2	+2.6	+3.2	+2.8
128	14. 4. »	6.0	+0.2	+0.2	—0.6	—	65	+ 5	+ 7	+ 5	—	6.2	—0.6	+3.1	+0.1
130	» » »	4.9	+0.6	+0.6	+0.2	0.0	80	— 3	+ 2	— 2	—	4.2	+0.2	+1.4	—1.6
A 54	29. 4. »	4.4	+0.4	—	+0.9	+0.9	71	+ 1	—	+ 1	+ 8	6.4	—2.4	+1.6	—
144	26. 5. »	4.9	+0.4	+0.6	+0.4	—	70	+ 8	+12	—	—	—	—	—	—
162	12. 10. »	4.6	+0.5	+0.7	+0.3	+0.5	72	+ 3	+ 7	— 5	+ 1	3.0	+1.0	+2.2	—0.8
163	» » »	5.0	+0.1	+0.2	+0.3	0.0	75	— 6	— 4	— 4	— 3	3.6	+1.2	+3.0	—0.8
257	26. 10. »	5.2	+0.2	—0.4	+0.3	—	71	+ 8	— 1	+ 3	—	3.1	+3.0	+2.9	—
277	» » »	5.6	—0.3	—0.2	—0.2	—	83	—10	—14	— 6	—	4.7	+0.3	+1.9	—
73	20. 11. »	5.3	0.0	—0.1	—0.2	—	79	—14	0	— 9	—	2.8	+2.2	+1.2	—
88	» » »	5.4	—0.1	—0.1	—0.2	—	78	—	—17	— 5	—16	3.0	+1.4	+0.6	—
95	23. 11. »	5.5	+0.6	+0.7	+0.3	—0.2	83	+ 7	— 3	+ 2	— 8	4.3	+1.0	+2.9	+1.3
136	» » »	5.2	+0.3	+0.3	+0.5	—0.3	80	—11	— 9	—10	—18	4.2	+0.2	+2.8	+0.2
73	7. 12. »	5.3	0.0	+0.2	—	—	83	—13	— 8	—	—	5.8	—1.7	+1.3	—
136	9. 12. »	5.2	+0.3	+0.2	—	—	74	+ 4	0	—	— 4	4.8	+1.3	+1.7	—
A329	» » »	5.1	+0.7	+0.5	—	—	75	+ 9	— 5	—	0	6.8	+2.7	—0.7	—
Mean		5.16 ±0.07	+0.31 ±0.07	+0.31 ±0.07	+0.16 ±0.09	+0.17 ±0.15	73.3 ± 1.6	+ 0.3 ± 1.9	—1.7 ± 1.5	—0.9 ± 1.5	—1.8 ± 2.4	4.41 ±0.29	+1.07 ±0.37	+1.87 ±0.30	+0.68 ±0.63
n		20	20	19	16	10	20	19	19	15	12	18	18	18	10

confirmed. As regards haemoglobin, the average daily deviations from the initial value are without statistical significance. The average increase in the percentage of reticulocytes, again, is statistically well confirmed on 1st and 2nd day, while the increase on the 3rd and 4th day falls within the limits of random variations.

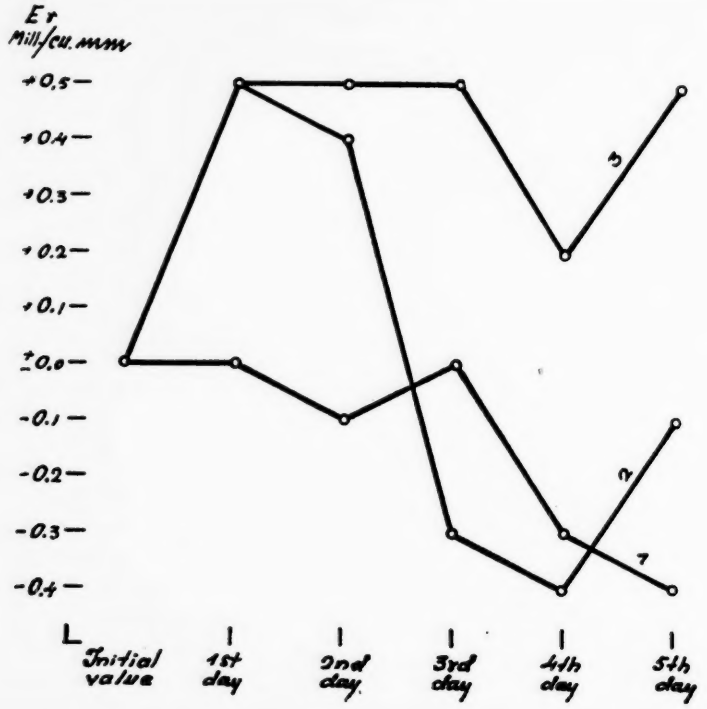

Fig. II_2. Effect of injection of plasma from the same donor (135) on the recipients' red cell count. 1. Plasma from blood subjected to normal pressure, 2. and 3. plasma from blood subjected to low pressure.

As stated before the effect of »lov pressure plasma» can be most adequately evaluated by a comparison to the effect of normal rabbits plasma. The comparison can be made in several ways, comparing for instance the effect of »low pressure» and »normal» plasma samples from the *same donor* on different recipients, or by comparing the *average* effect of »low pressure» and »normal» plasma. The effect of »low pressure» and »normal» plasma from the same donor is illustrated in Fig. II_2. (Two recipients received »low

pressure» plasma, one »normal» plasma.) It is seen that »normal» plasma caused a slight decrease in the recipients red cell count, while »low pressure» plasma called forth a considerable increase in the recipients' red cells lasting in one case for 2 days and in the other case until the end of the experiment (5 days).

The comparison between the average effect of »low pressure» and »normal» plasma as regards the red cells is illustrated in Fig. II_3. The average initial values on the day of injection for both groups

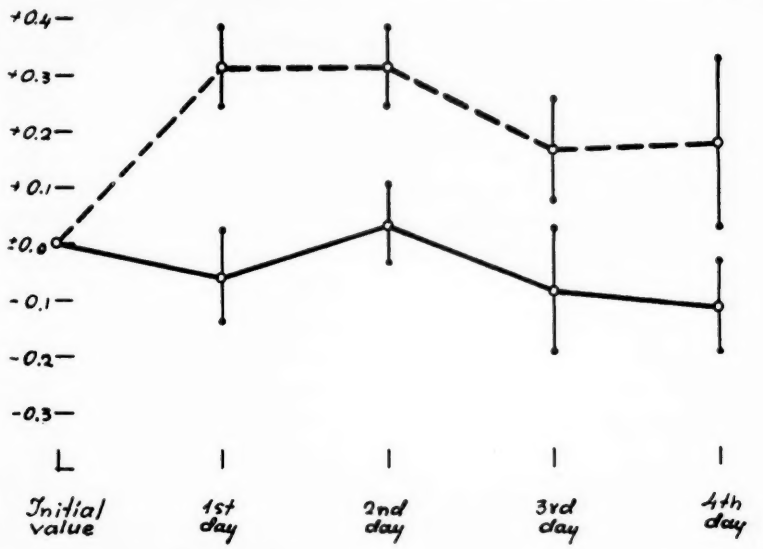

Fig. II_3. Average effect of »normal» and »low pressure» plasma on the red cell count of rabbit. Normal o——o, Low pressure o— — —o.

are placed on the common 0-point of the two curves. Each point on the curves represents the mean deviation from the initial value on the day indicated below on the abscissa. The mean errors of the means are drawn as vertical lines. It is seen clearly that the two curves differ markedly at points corresponding to the 1st and 2nd day after injection. On the later days the difference is less evident. Calculation of the t-values for the significance of the difference of the two series gives, for the 1st day, $t = 3.0$ and for the 2nd day, $t = 2.1$, in other words, on the 1st day the difference can be regarded as statistically confirmed ($P < 0.01$), and, on the 2nd day, as statistically probable ($P < 0.05$). A similar

comparison between the average reticulocyte reaction after injection of »normal» and »low pressure» plasma shows that the difference between the average values here, too, is statistically probable on the 1st day and significant on the 2nd day, the t-value being for the 1st day 2.0 ($P < 0.05$) and for the 2nd day 5.3 ($P < 0.001$).

To summarize: the injection of »low pressure plasma» causes a moderate increase in the recipient's red blood count, which, when compared to the effect of normal plasma injection, is statistically significant as regards the red cells and reticulocytes.

The erythropoietic Activity in Blood Containing High and Low Percentage of Reticulocytes after Exposure to Low Pressure. — The experiments presented above seem to prove clearly enough the presence of some erythrocytosis promoting factor in plasma from blood subjected to lowered atmospheric pressure. After the present data were collected, however, attempts of other investigators as well as of the present writer to reproduce these results have largely failed. This led the writer to consider the possible factors which might be responsible for the differing results. Such a factor was seen in the reticulocyte level of the test rabbits. As noted before (see Methods, Part II) the percentage of reticulocytes in the present test subjects was at the upper limit of the normal range. On the basis of results given in table II_2, there is, however, no significant difference between the reaction of recipients with high and low initial reticulocyte count to the injection of »anoxic» plasma. Accordingly, the percentage of reticulocytes of the recipients can hardly account for the inconsistent results. The reticulocyte percentage of the donors, now, remains to be considered. As the reticulocytes of the donors' blood were not enumerated before the experiments, nothing can be said of the individual donors' reticulocyte percentage. The general level of the donors' reticulocytes, on the other hand, can be estimated on the basis of the recipients' reticulocyte count: as mentioned in the methods, the donors and the recipients were mostly of the same breed, and were living in identical conditions. Besides, animals used previously as recipients in some cases later served as donors. Accordingly, the reticulocyte level of the donors probably corresponded to that of the recipients. Later attempts of the writer, on the other hand, to reproduce these results were performed on animals with a lower reticulocyte level.

In order to test the rôle of the reticulocyte percentage of the donors' blood in the formation of the active erythropoietic substance, the following experiments were planned.

Six rabbits were rendered anaemic by removing 20—40 cc of blood from the heart. When the animals showed signs of regeneration — high reticulocyte percentage — blood was again drawn by heart punctures, 11 samples altogether. These »high reticulocyte» samples were either subjected to lowered atmospheric pressure for 4 hours, and thereafter centrifugalized, or centrifugalized immediately; 2.5—4.0 cc of plasma were injected into normal recipients. In a few cases the »high reticulocyte» sample was devided into two, one part was subjected to lowered atmospheric pressure, while the other part was centrifugalized and injected without delay.

It was considered appropriate to inject the plasma samples in the control group without delay, because in an earlier group of control experiments it was already shown that blood gains no erythropoiesis promoting activity during exposure to normal atmospheric pressure (Table II_1). On the other hand, Klingelhöffer claims that initially active plasma loses the activity when exposed to atmospheric air. Thus, the exposure might also abolish the activity of an initially active blood sample. The possibility that blood containing a high percentage of reticulocytes might gain erythropoietic activity while standing in open air was still not excluded, this, however seemed unlikely, as the donor animals of the former control group probably also had a relatively high reticulocyte level, as noted before.

As a control to the »high reticulocyte low pressure plasma» 6 samples of normal rabbits' blood were subjected to lowered pressure, and, after centrifugalization, the plasma (»low reticulocyte low pressure plasma») was injected into normal recipients.

The reticulocyte count of the normal donor rabbits ranged from 2.2—4.9%, and that in the »anaemized low pressure» group from 4.2 to 10.0%. As the lowest value in the latter group (4.2%) differs considerably from the next one (5.8 %) and falls within the range of normal reticulocyte values in the present test subjects, this rabbit will be treated in the following account as a »low reticulocyte» donor. In the »high reticulocyte untreated» group the reticulocyte count varied from 4.8 to 13.0% (Table II_4), being essentially of the same magnitude as in the »high reticulocyte low pressure» group.

TABLE II_3

EFFECT OF PLASMA FROM RABBIT BLOOD, SUBJECTED TO LOW PRESSURE, CONTAINING OVER 5 PERCENT OF RETICULOCYTES, ON RABBITS' RED CELL COUNT

Date	Donor	R % of Donor	Recipient	Initial Er Count of Recipient	Deviation from Initial Er Count				
					1st Day	2nd Day	3rd Day	4th Day	5th Day
19. 12. 50	205	5.8	206	5.2	+0.1	—0.3	+0.2	—0.1	—
» » »	»	»	207	4.8	+0.5	+0.6	+0.7	+0.7	—
8. 1. 51	322	7.9	204	4.5	+0.4	+0.5	+0.1	+0.1	—
» » »	»	»	206	5.0	+0.1	—0.2	—0.1	—0.1	—
» » »	»	»	207	4.7	+0.3	0.0	—0.1	0.0	—
16. 1. »	322	6.4	173	4.5	+0.6	+0.7	+0.3	—0.2	—
» » »	»	»	316	4.8	+0.2	+0.3	+0.3	+0.5	—
23. 1. »	323	10.0	320	4.5	+0.1	+0.5	+0.5	0.0	0.0
» » »	»	»	A954	5.1	+0.5	+0.6	+0.1	+0.1	0.0
» » »	»	»	271	5.1	+0.4	+0.5	0.0	—0.3	—0.4
» » »	»	»	272	4.8	+0.5	+0.4	—0.1	—0.2	—
12. 2. »	325	8.6	204	4.6	+0.1	+0.6	+0.4	+0.1	+0.2
» » »	»	»	272	4.8	+0.5	+0.6	+0.2	+0.1	+0.1
21. 2. »	325	6.8	A962	5.1	+0.3	—0.5	—0.6	+0.3	—
» » »	»	»	278	5.0	+0.1	—0.1	0.0	—	+0.1
7. 3. »	317	9.8	215	4.9	+0.4	+0.3	+0.9	—	+0.7
» » »	»	»	217	5.1	+0.5	+1.0	+0.7	—	+1.0
» » »	»	»	219	5.4	—0.8	+0.3	—0.7	—	+0.1
17. 3. »	320	9.3	1000	5.9	—	+1.6	+0.6	—	—
Mean				4.94 ±0.07	+0.27 ±0.06	+0.39 ±0.11	+0.18 ±0.09	+0.07 ±0.05	+0.20 ±0.12
n				19	18	19	19	14	9

15 recipient rabbits were given injections of »high reticulocyte low pressure» plasma (reticulocyte percentage 5.8—10.0), some of them twice so that 19 injections were given altogether. The injection caused, with a few exceptions, an increase as great as 0.5 Mill./cu.mm in the erythrocyte count as compared to the initial value. In the haemoglobin values there were no consistent changes, but fairly considerable deviations in both directions equally.

As the haemoglobin changes, when statistically treated, indicate no constant changes in either direction, only the changes in the erythrocyte count of the recipients will be presented (Table II_3). From the bottom line it is seen that the mean deviations from the initial values are statistically confirmed on the 1st and 2nd day,

TABLE II_4

EFFECT OF PLASMA FROM RABBIT BLOOD, SUBJECTED TO LOWERED PRESSURE, CONTAINING LESS THAN 5 PERCENT OF RETICULOCYTES, ON RABBITS' RED CELL COUNT

Date	Donor	R% of Donor	Recipient	Initial Er Count of Recipient	Deviation from Initial Er Count				
					1st Day	2nd Day	3rd Day	4th Day	5th Day
16. 1. 51	182	4.3	208	4.8	+0.1	+0.1	—0.3	—0.1	—
» » »	»	»	317	4.6	+0.1	+0.1	+0.2	—0.7	—
* 27. 1. »	323	4.2	208	4.8	—	+0.1	+0.2	0.0	—
» » »	»	»	A951	5.0	+0.4	—0.3	0.0	—	—
5. 2. »	325	3.8	213	4.8	—0.1	—0.5	—0.3	—0.2	—
» » »	»	»	317	4.5	—0.2	—0.2	+0.1	+0.2	—
» » »	»	»	A954	5.1	—0.1	0.0	0.0	—0.1	—
» » »	»	»	271	4.8	+0.1	+0.1	+0.1	+0.1	—
12. 2. »	316	4.4	207	4.6	+0.2	—0.1	—0.1	—0.2	—0.1
» » »	»	»	A951	4.7	+0.5	+0.5	+0.2	+0.1	+0.1
21. 2. »	317	3.7	1000	5.8	+0.3	+0.7	+0.3	+0.3	—
» » »	»	»	A917	5.3	—0.2	+0.2	—0.3	—0.2	
» » »	»	»	A997	4.4	+0.3	+0.1	+0.6	—	+0.3
7. 3. »	320	4.9	214	6.3	—0.1	—1.0	0.0	—	—
» » »	»	»	216	4.6	+0.4	—0.2	—0.4	—	+0.4
» » »	»	»	218	6.8	+0.2	+0.3	—0.5	—	+0.3
17. 3. »	321	2.2	A997	4.9	—	—0.1	+0.3	—	—
» » »	»	»	222	5.4	—	—0.4	+0.1	—	—
Mean				5.07 ±0.14	+0.13 ±0.07	—0.03 ±0.08	+0.01 ±0.07	—0.07 ±0.06	+0.20
n				18	15	18	18	11	5

* anaemic donor

and that the mean deviation on the 3rd day borders on statistical probability, whereas on the 4th day there is no difference from the initial value.

In table II_4 the changes in the recipients' red cell count after injection of plasma from »low reticulocyte» blood kept under lowered atmospheric pressure are given (14 rabbits, 18 injections altogether). Only a few recipients responded to the injection with a marked increase of erythrocytes, while in others only slight deviations in both directions were observed. The mean values of the daily deviations and the mean errors of the means show that the mean increase on the 1st day borders on statistical probability, while on the later days the values do not differ

TABLE II_5

EFFECT OF PLASMA FROM UNTREATED BLOOD, CONTAINING A HIGH PERCENTAGE OF RETICULOCYTES, ON RABBITS' RED CELL COUNT

Date	Donor	R % of Donor	Recipient	Initial Er Count of Recipient	Deviation from Initial Er Count			
					1st Day	2nd Day	3rd Day	4th—5th Day
8. 12. 50	322	4.8	325	5.0	+0.1	—	—0.2	+0.1
» » »	»	»	206	5.0	—0.1	—	+0.2	—0.2
9. 12. »	205	13.0	207	4.8	—	—0.6	—	—0.7
» » »	»	»	204	4.9	—	0.0	—	—0.4
8. 1. 51	322	7.9	325	5.0	0.0	—0.3	—0.1	—0.5
16. 1. »	322	6.4	213	4.7	+0.4	+0.1	—0.2	—0.3
Mean				4.90 ±0.05	+0.10 ±0.10	—0.20 ±0.16	—0.08 ±0.08	—0.33 ±0.11
n				6	4	4	4	6

significantly from the initial value. In haemoglobin no significant changes were encountered.

5 normal rabbits served as test animals to control the effect of anaemic, untreated plasma; one of these recipients was injected twice (325). None of these showed, after the injection, any significant changes in the red cell count, nor in haemoglobin. As there thus was no essential difference between the effect of plasma from an anaemie donor with high resp. normal reticulocyte count, the material is presented as a whole in Table II_5.

When comparing the erythropoietic activity which high reticulocyte blood resp. low reticulocyte blood gains when exposed to lowered barometric pressure, the rôle of the donors' reticulocyte count is evident. While »high reticulocyte low pressure» plasma causes a statistically confirmed increase in the recipients' erythrocyte count, after the injection of »low reticulocyte low pressure» plasma the mean erythrocyte deviation from the initial value is statistically probable only on the 1st day. The comparison is represented in Fig. II_4, two upper curves. (The points on the curves represent the mean deviations from the initial value on 1st—4th days, the mean errors of the means are drawn as vertical lines.)

When the average erythrocyte deviations in the high and low reticulocyte low pressure groups are compared by means of calculating the coefficient t, it is seen that, while the difference of the

two groups on the first day is without significance ($t = 1.2$, $P > 0.1$), on the second day the difference is statistically confirmed ($t = 2.8$, $P < 0.01$).

The group of recipients which received injections of »high reticulocyte untreated» plasma served as control to find out whether plasma from anaemic, regenerating blood might promote erythrocytosis, and whether the effect shown in Table II_3 might thus not be due to anaemization as such rather than to the exposure to low

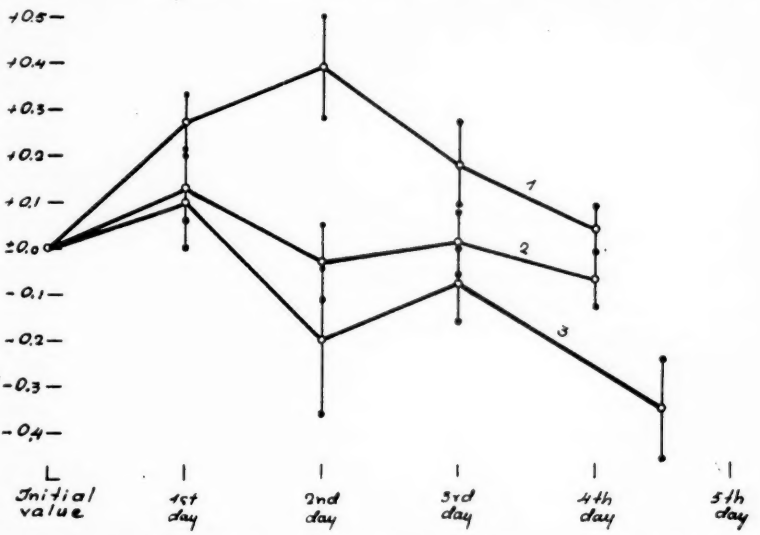

Fig. II_4. Average effect of plasma from »high reticulocyte» blood (1) and from »low reticulocyte» blood (2), subjected to low pressure, and from untreated »high reticulocyte» blood (3), on the recipients' red cell count.

pressure. Table II_5 and Fig. II_4 (lowest curve) indicate that anaemization as such is not a sufficient stimulus for formation of erythropoietic substances in concentrations strong enough to promote erythrocytosis when injected into another animal, though it, in most cases, brings about signs of regeneration in the donor animal.

To summarize: the present experiments indicate that anaemic blood, which, as such, is erythropoietically inactive, may gain erythrocytosis promoting activity when subjected to lowered atmospheric pressure. On the other hand, normal blood when exposed to low pressure, gains no, or only a slight erythrocytosis promoting activity.

This result suggests that two factors are necessary for the formation of an active, transferable haemopoietic substance in blood in vitro: 1. a state of increased regeneration of the blood and 2. exposure to lowered atmospherie pressure.

The Oxygen Content of Blood Subjected to Low Pressure. — On the basis of the results presented above, the assumption that the erythropoietic activity in blood kept under lowered atmospheric pressure in vitro would be due to formation of anoxic haemopoietin seems natural. However, the conditions in the present experiments are, in a very important point, different from those in an anoxic organism. In a living organism, oxygen is continuously used by the tissues, and replaced by oxygen taken up from the alveolar air. If the oxygen uptake of the arterial blood is not sufficient to balance the oxygen consumption, anoxaemia and tissue anoxia follow. In blood in vitro, the oxygen consumption is negligible (Q_{O_2} of defibrinated rabbit blood between 25 and 38° C ranges from —0.22 to —0.062, Ponder 1948, p. 358), and thus the decrease in the oxygen content would only be dependent on the oxygen pressure gradient between blood and the surrounding gas mixture, and on the equilibration between the blood and the gas.

To decide whether the oxygen content of the blood was influenced by lowered atmospheric pressure, the oxygen content was determined from 10 samples of rabbit blood after exposure of 1—4 hours to normal atmospheric pressure, and to a pressure of 20—300 mm Hg. Before the analysis the »normal» as well as the »low pressure» samples were covered with paraffin in order to avoid oxygen uptake from the atmospheric air. A contact between the »low pressure» sample and atmospheric air could not, however, be avoided for a short while, when the sample was removed from the low pressure chamber.

The results of the oxygen determinations are given in Table II_6. It is seen that in most cases the values in blood exposed to low pressure are slightly lower than in blood exposed to normal pressure. The average difference is, however, of a considerably smaller magnitude than that observed during venous obstruction (Table I_4), or in subjects breathing rarefied air. It is further noted that the oxygen values in the »normal pressure» group show rather large variations. This might indicate that a full equilibration of the

TABLE II_6

OXYGEN CONTENT OF BLOOD AFTER EXPOSURE TO NORMAL AND LOW PRESSURE

No. of Sample	Vol. % of O_2		Difference
	After Exposure to Normal Pressure	After Exposure to Lowered Pressure	
1	10.2	9.6	—0.6
2	14.6	14.5	—0.1
3	12.5	8.9	—3.6
4	11.8	11.7	—0.1
5	15.5	15.4	—0.1
6	17.7	15.5	—2.2
7	10.4	10.6	+0.2
8	16.0	15.0	—1.0
9	11.5	10.9	—0.6
10	13.0	11.5	—1.5
Mean	13.3 ± 0.9	12.4 ± 0.9	—0.96 ±0.35
n	10	10	10

blood with the atmosphere has not taken place, and thus, full equilibration in the low pressure group could not be expected.

The oxygen content in blood subjected to low pressure is only slightly lower than in blood subjected to normal pressure.

DISCUSSION OF PART II

The present results suggest that inactive blood gains, in some cases, erythropoietic activity when subjected to a reduced barometric pressure of 10—400 mm Hg. The activity of the »low pressure» plasma is dependent on the state of blood regeneration of the donor animal; blood containing a high precentage of reticulocytes gaining, during similar exposure, a higher activity than blood with a normal or low reticulocyte percentage. During the exposure the oxygen content of the blood is lowered only slightly.

When attempting to interpret these results, the most interesting questions are: 1. does blood kept in vitro under decreased oxygen pressure represent a case of anoxic haemopoietin formation, and 2. which is the primary cause of formation of the active substance. Neither of these questions can be answered definitely either positively or negatively; some discussion may yet be allowed.

The similarity of reaction of the recipient animal to the injection of plasma from anoxic animal, resp. from »low pressure» blood favours the conception that the stimulation mechanism is the same in both cases. In the present test subjects, erythrocytosis and reticulocytosis appeared on the 1st—2nd day, and the values were levelled again on the 3rd—5th day. The same kind of reaction was observed after injections of plasma from anoxic organisms in test animals partly of the same breed and living in the same conditions (Bonsdorff and Jalavisto). The present reaction is also of the same magnitude and duration as that observed in human subjects after venous congestion (see Part I).

If the erythropoietic activity in blood subjected to low pressure in vitro is interpreted as due to anoxic haemopoietin formation, the changes in the oxygen content and oxygen pressure are questions of chief interest. In the earlier experiments concerning anoxic haemopoietins the donors doubtlessly suffered from arterial anoxaemia — they were subjected to atmospheric pressures of 300—400 mm Hg for 4—6 hours. In human experiments (Part I) local congestion also caused a marked decrease in the venous oxygen saturation. In the present experiments, on the other hand, the oxygen content of the blood was shown to suffer only a very slight decrease during the exposure. This leads to the doubt, whether equilibration between blood and air has taken place. There is, indeed, no evidence of full equilibration. To ensure full equilibration, gas is usually bubbled through the blood, or the sample is shaken for a long period. In the present experiments such measures were avoided because of the danger of haemolysis. Another factor which was not favourable for equilibration in the present experiments was the relatively small contact area between blood and air, as the samples in most cases were exposed in test tubes of 3—4 cm diameter. The use of larger test tubes, which would have secured a better ratio between surface and volume, was impossible because a thorough mixing of the blood sample during the oxygen analysis requires a deep layer of blood, and, on the other hand, the amount of blood obtainable for each experiment was limited.

However, imperfect equilibration is not the only possible explanation for the result of the oxygen analysis. It must be born in mind that not only changes in oxygen partial pressure, but also shifts in the haemoglobin dissociation curve influence the oxygen

content of the blood. If the haemoglobin dissociation curves at p_{CO_2} 40 mm Hg and at p_{CO_2} 3 or 0 mm Hg are compared (Drabkin 1949, p. 45) it is seen that the shape of the curve corresponding to low carbon dioxide pressures is flat until fairly low oxygen pressures, whereas at the physiological carbon dioxide pressure the slope of the curve begins at considerably higher oxygen pressure. Thus, if the atmospheric pressure were reduced to, say, 100 mm Hg, corresponding to an oxygen partial pressure of approximately 20 mm Hg, the oxygen saturation in low carbon dioxide pressure would still be approximately 85 per cent, and even at an oxygen pressure of 10 mm Hg would not fall considerably below 60 per cent. This means that, at low carbon dioxide pressure, there is no marked decrease in the oxygen saturation of haemoglobin until the atmospheric pressure is reduced below 100 mm Hg. Thus, the lack of a marked decrease in the oxygen saturation may be partly attributed to the shift to the left of the haemoglobin dissociation curve, and not be solely due to imperfect equilibration. Equilibration to some degree must be assumed on the basis of the fairly constant, though slight decrease in the oxygen content, which was observed after the exposure; and the decrease in the oxygen pressure probably is of a greater magnitude because of the effect of the washing out of carbon dioxide on the oxygen dissociation curve. — The amount of dissolved oxygen in the plasma, which would have given a true picture of the oxygen pressure, could not be determined because of the lack of a sufficiently precise method.

On the basis of the above considerations it is not altogether unlikely that the oxygen pressure in the blood — dissolved oxygen — has suffered a decrease in the present experimental conditions. On the other hand, the dissolved and not the combined oxygen is essential for some metabolic processes in the red cells. Thus, the oxygen pressure influences methaemoglobin formation which is maximal at 19.7 mm Hg (Brooks 1931, 1935, 1948, Legge 1942) and the normal glycolysis in erythrocytes, according to Granick (1949), is dependent on the oxygen available. Some correlation between metabolic processes in erythrocytes and erythropoiesis is suggested, on the other hand, by the observation of Duesberg and Koll (1931) that therapeutically active liver substances form methaemoglobin.

The present experiments suggest that a high percentage of

reticulocytes in the blood subjected to low pressure is essential for the formation of the active substance. It may be remarked that plasma from anaemic, regenerating blood has long been recognized as a stimulus for erythropoiesis, and thus, the result of the present writer that anaemic blood is not effective without exposure, might seem unlikely. As an answer it can only be remarked that the removal of blood in the present material, though sufficient to cause marked regeneration in the donor animal, may not have been sufficient to give rise to the formation of the haemopoietic factor in concentration high enough for 2—4 cc to stimulate the erythropoiesis of the recipient. The importance of the donor animals regenerative state for the formation of the active substance, is, on the other hand, in good accord with some earlier observations. Gibelli reports that the injection of serum from a normal test animal, taken during high haemopoietic activity due to a previous injection of anaemic serum, causes a pronounced erythrocytosis in an anaemic or a normal »second recipient». The experiments of Oliva and Frascarelli (1946) and of Oliva, Chinnini and Tramontana (1949) on human patients in a regenerative state point in the same direction: 20 cc of plasma from liver treated pernicious anaemia patients or other regenerating anaemia patients injected into normal men caused a reticulocyte increase in these while injection of normal human plasma was ineffective. Thus, it seems evident that the regeneration of blood in the donor organism and the formation of haemopoietins are closely correlated, and in view of the results of Gibelli, and of the writers observation that blood from an anaemic rabbit which showed no signs of regeneration did not gain erythropoietic activity when exposed to lowered pressure, (donor 323, Table II_4), the regenerative state of the donor organism seems to be more important for the formation of the active substances than anaemia as such.

A discussion on the relation of the active substance formed in regenerating blood kept under low atmospheric pressure to other haemopoietic substances (reticulocyte ripening substance of Plum, liver compounds, pteroylglutamic acid etc.) would be interesting, but probably might lead too far from the problems of the present study.

As regards the nature of the erythrocytosis in the present experiments, approximately the same considerations hold good as pre-

sented in Part I. It is most likely, though not definitely proved, that the increased number of circulating erythrocytes is due to increased erythropoiesis. The stimulation would thus be transmitted by some humoral factor, which is probably identical with Carnot's anoxic haemopoietin.

The possibility, suggested by the present results, that the humoral bone marrow stimulating agent should be formed in blood itself is an interesting prospect. In physiological thinking, such an arrangement would be most adequate: the erythropoietic organ would be informed of increased need of red cells, regardless whether the need were general, due to general anoxia or increased oxygen consumption, or local — impaired circulation or high oxygen consumption in part of the organism.

SUMMARY

In the present experiments the formation of erythropoietically active substances in stagnant blood and in blood subjected to lowered atmospheric pressure in vitro has been studied.

In the first part local venous stagnation in an arm or leg was induced by means of a pneumatic cuff, inside which a pressure of 40—60 mm Hg was maintained for an hour. This treatment was applied to 25 adult subjects and 30 children. On a few days following the stagnation an increase in the peripheral red cell count amounting to 0.5 Mill./cu. mm and a less constant rise in the haemoglobin value was observed. In children the percentage of reticulocytes was also increased on the 1—3 days following the treatment. The increase in the erythrocyte count in children and adult subjects, as well as the increase in the childrens' reticulocyte percentage was statistically confirmed.

The percentage of oxygen saturation before and at the end of stagnation was estimated in 10 adults. There was a statistically confirmed decrease in the oxygen saturation during the stagnation. The oxygen capacity of the blood was slightly increased during the stagnation, and the erythrocyte count performed on 5 subjects from the capillary blood before the stagnation and 10 minutes after the onset of the stagnation suggested that there was a slight haemoconcentration during this period.

In 15 adult subjects the serum bilirubin, the mean erythrocyte diameter, the mean erythrocyte thickness and the packed cell volume from the venous blood and the number of leucocytes from the capillary blood were determined before and at the end of congestion. No significant changes in these values were observed. The negative result as regards the bilirubin as well as the mean erythrocyte diameter and thickness values was interpreted as indicating that no significant haemolysis occurred during stagnation.

The nature and the mechanism of the peripheral erythrocytosis are discussed. It is concluded that the erythrocytosis is probably due to a general stimulation of erythropoiesis in the bone marrow, transmitted by some humoral agent formed in the area of congestion.

The second part comprises experiments on the formation of erythrocytosis promoting factors in blood subjected to lowered atmospheric pressure in vitro.

In the first experimental group 18 normal rabbits received injections of plasma separated from blood kept under a barometric pressure of 10—400 mm Hg, and as a control for this group 7 rabbits were injected with plasma from blood kept under normal atmospheric pressure. When comparing the results in the experimental and control group a definite difference was observed. While the test animals of the control group showed no significant changes in the peripheral blood picture, in the animals of the experimental group the injection was followed by a rise in the red cell count and reticulocyte percentage, which could be statistically confirmed.

As the test subjects were rabbits with a relatively high reticulocyte percentage, in a further experimental series blood containing high and low percentage of reticulocytes was subjected to low barometric pressure, and the erythrocytosis promoting effect brought about by the exposure was compared by injecting plasma samples into normal test rabbits, 15 animals received plasma from blood rich in reticulocytes, and 14 received plasma from blood poor in reticulocytes. Comparison between the two groups showed that the erythrocytosis promoting effect in the former group was considerably stronger, for the recipient rabbits reacted to the injection with a statistically significant rise in the peripheral erythrocyte count, while in the latter group the increase in the erythrocyte count was less, and statistically insignificant.

As a further control, plasma from untreated anaemic blood, containing a high number of reticulocytes was injected into 5 test rabbits. As these showed no significant rise in the red cell count after the injection, it was considered that anaemic blood with a high reticulocyte percentage does not always contain erythrocytosis promoting factors in concentration high enough to bring about erythrocytosis in the recipient animal.

The determination of the oxygen content of rabbit blood after exposure to normal and low pressure performed using 10 samples showed that the oxygen content was only slightly lower after the exposure to low pressure.

In the discussion of the second part the mechanism of formation of the erythropoietic factor in blood subjected to low pressure in vitro is treated. It is suggested that, though the difference in the oxygen content of the blood after exposure to normal and low atmospheric pressure is slight, the oxygen pressure might yet be considerably decreased, as the shift to the left in the oxygen dissociation curve caused by the washing out of carbon dioxide lowers the ratio oxygen pressure/oxygen content of the blood.

The correlation between the anoxic haemopoietin of Carnot and the present erythrocytosis promoting factor is discussed. It is suggested that erythrocytosis observed after local venous congestion, as well as erythrocytosis due to injection of plasma from blood subjected to low atmospheric pressure represent cases of stimulation of erythropoiesis by anoxic haemopoietin.

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